

ABSTRACT

Title of Thesis:

ENHANCEMENT OF THERMAL
INACTIVATION OF FOODBORNE
PATHOGENIC BACTERIA AT MILD
HEATING TEMPERATURES WITH
INCLUSION OF BUTYL PARABEN
AND THE APPLICATION ON FOODS

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Thermal processing is widely used in food industry to ensure the microbial safety, however, there is increasing demand on reducing the processing temperature and duration. This study specifically focused on mild heating temperatures ($<60\text{ }^{\circ}\text{C}$) with inclusion of low level ($\leq 125\text{ ppm}$) of the approved preservative butyl-parahydroxy-benzoate (BPB). In a BHI model matrix, four pathogens were studied with submerged coil apparatus: *Cronobacter sakazakii* 607, *Salmonella enterica* serotype Typhimurium, attenuated *Escherichia coli* O157:H7 and *Listeria monocytogenes*. The results indicated that low concentrations of BPB combined with temperatures $< 60\text{ }^{\circ}\text{C}$ achieved 5 – 6 log reductions in less than 15 minutes with tested gram-

negative microorganisms, whereas reductions without BPB were only 1 – 2 logs. We further extended the study to food applications: powdered infant formula, non-fat dry milk, and apple juice. The results indicate BPB will be inhibited by proteins, but apple juice is a suitable application.

ENHANCEMENT OF THERMAL INACTIVATION OF FOODBORNE PATHOGENIC
BACTERIA AT MILD HEATING TEMPERATURES WITH INCLUSION OF BUTYL
PARABEN AND THE APPLICATION ON FOODS

By

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Foreword

It is extremely tough to summarize my experience of the Master study in the past two and half years. I spent the first year composing a proposal, familiarizing myself with all of the instruments, and experimentally proving what I initially proposed was not applicable. Then I spent the second year investigating other possibilities of applying the proposed treatment and the reasons of inapplicable cases. After I got a solid understanding, I changed the research direction and finally found a proper model for my proposal during my last half year of Master study. My advisor, Dr. Robert L. Buchanan, always says, “We learned more from mistakes.” Indeed, I made numerous mistakes during my Master study in my proposal, research design, and experiment analysis, etc. And I also incredibly improved in my knowledge by learning from these mistakes. During these learning processes, Dr. Buchanan served as a perfect advisor and mentor for me, and I truly appreciate it. His wisdom helped me to identify the technical issues; his patience and kindness encouraged me to build up my confidence; his optimism also influenced my life principles. Closely working with Dr. Buchanan, Dr. Tikekar, and Qiao Ding is a precious experience for me, not only for learning the knowledge, but also for fostering beneficial research habits. As a graduate student, serving twice as teaching assistant for Food Microbiology Lecture & Lab helped me learn how to utilize my knowledge and teach other people. All of these experiences created an essential transition in my life. They made what I am now and helped me to determine how I would like to move forward. As looking forward, I wish my advisor Dr. Buchanan will enjoy his retire life, and I hope I can essentially get to where I want to be.

Dedication

Here I dedicate this work and my efforts to all the people who helped, guided, encouraged, and supported me during the past two and half years. To my loving parents, Wu and Houyu, my reliable lab mates Qiao and Ruth, and my lovely friends Sara, Qing, Heejin and so on. This is for you all.

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Chapter 1: Introduction

1.1. Background

While thermal processing is widely used in the food processing industry to ensure microbial safety, there can be negative consequences associated with thermal processing. Excess thermal processing can result in loss of heat sensitive nutrients, the formation of thermally generated toxicants, and both desirable and undesirable changes in flavor and texture. Most of these changes in food characteristics are irreversible and might affect the overall attractiveness of the final products. Additionally, high temperature processing incurs increased financial costs. All of these issues indicate the necessity of conducting thermal processing at milder temperatures (e.g., $< 60\text{ }^{\circ}\text{C}$). However, to achieve such a goal without long processing times, a means for reducing the thermal resistance of foodborne pathogenic microorganisms would be needed. Previous studies have suggested that specific classes of naturally occurring flavor compounds and approved food additives can disrupt the bacterial cell membranes and act synergistically with mild heating to enhance the inactivation of foodborne pathogens by decreasing the microorganisms' thermal resistance (Milillo et al., 2011; Gurtler et al., 2012; Espina et al., 2013; Shi et al., 2015; Ruan and Buchanan, 2016). This, in turn, allows the thermal inactivation to be efficiently conducted at lower temperatures. In our laboratory (Ruan and Buchanan, 2016), it was proved that the addition of low levels of various esters of parahydroxy-benzoic acid (i.e., parabens, parahydroxy-benzoates) enhanced the inactivation of a thermally resistant strain of *Cronobacter sakazakii* 607 at a mild heating temperature ($58\text{ }^{\circ}\text{C}$), with the effectiveness being dependent on concentration of the compound and the length of the carbon side chain. Parabens are a class of widely used preservatives and emulsifiers in cosmetics, foods, and pharmaceutical

products. In food industry, they are used as emulsifiers in beverages and alcohols as well as preservatives in other food products. Possible bactericidal and fungicidal mechanisms include disrupting both membrane transport and the electron transport system (Freese et al., 1973), disrupting the membrane integrity and fluidity, and disrupting key enzyme activities (Davidson, 2005)

Most of available paraben studies have focused on model systems rather than actual food applications. However, foods are complex in terms of ingredients, processing techniques and the characteristics of food itself. This raises the possibility that these compounds could act differently in real foods than in model systems. Parabens could potentially interact with chemical constituents of foods during processing to modify the compounds' antimicrobial activity. As a result, applying paraben treatment to a wider range of foodborne pathogens and examples of foods products would help determine whether this or related classes of compounds could be effectively used to enhance thermal processing at mild processing conditions. With this consideration, the representative foods selected were powdered infant formula, non-fat dry milk, and apple juice.

1.2. Hypothesis and Project Goals

Based on the previous results collected by Dr. Buchanan's research group, combined paraben and mild heating treatments effectively enhanced thermal inactivation of *C. sakazakii* in BHI as a model. The current study hypothesized that this combined treatment could be productively inactivate other foodborne pathogenic microorganisms: *Escherichia coli* O157:H7, *Salmonella enterica* serotype Typhimurium, and *Listeria monocytogenes*. It was further

hypothesized that is combined treatment would all these four microorganisms to be thermally processed at temperature $< 60\text{ }^{\circ}\text{C}$).

If the hypothesis was correct and enhanced thermal inactivation was observed with all four bacteria in a BHI model system, we further hypothesized that low levels of butyl parahydroxybenzoate (BPB) would enhance the thermal inactivation in food products during thermal processing at temperatures below $60\text{ }^{\circ}\text{C}$. As explained later in the study, this hypothesis was tested in powdered infant formula and non-fat dry milk as dry and rehydrated foods, and apple juice as a liquid food.

1.3. Study Approach

The general study approach started with the inoculation of target microorganism into the testing matrices: BHI, powdered infant formula, non-fat dry milk, and apple juice. Low level of BPB was then supplemented into the inoculated substance, after which the heat treatment was applied using the designated instrument. The survivor cells were collected at designated times with and without BPB or thermal treatment, and then the cells were surface plated on tryptic soy agar (TSA) with spiral plater and enumerated with a plate counter. After enumeration, survivor curves were plotted and compared using Microsoft Excel. This general principle served as the overall study approach on investigating the microbial heat resistance and potential synergism in this project. Detailed methodology will be discussed in Chapter 3.

Chapter 2: Literature Review

2.1 The Problem

Thermal processing is widely used in food industry. There are two major temperature categories employed in thermal processing: pasteurization and sterilization. The basic purposes for the thermal processing of foods are to reduce or destroy microbial activity, reduce or destroy enzyme activity and to produce physical or chemical changes to make the food meet a certain quality standard, (e.g. gelatinization of starch, denaturation of proteins), to produce an edible food. There are a number of types of heat processing employed by the food industry (Safefood 360, Inc., 2014). However, such processes often have a detrimental effect on product sensory quality (Bean, et al., 2012). Some heat sensitive nutrients will be lost or inactivated such as vitamins and amino acids. Heat will also initiate other chemical reactions that will form undesirable compounds. For example, acrylamide will form in heated food, even at temperatures lower than 100 °C (Becalski et al., 2011). The disadvantages of thermal processing also include high capital costs (Safefood 360, Inc., 2014).

To overcome the disadvantages associated with thermal processing, developing the processing techniques at mild heating temperatures is a primary alternative (Timmermans et al., 2011). Based on the consumer behaviors, it is a trend that consumers prefer foods that have minimal processing or reduced levels of chemical preservatives (Allenda et al., 2006). Minimal processing includes heating the food at lower temperatures and/or for shorter period, however, both of the cases will introduce microbial safety risks. Therefore, it is necessary to enhance the microbial inactivation or reduce the microbial resistance against the specific processing technique. The general consumer demand for reducing chemicals additives suggests the need to discover novel applications of processing aids or preservatives.

There are some foods driving public attention in relation to maintaining high levels of overall safety, minimizing the processing techniques, and reducing the level of chemicals used in foods. One example is infant formula. Powdered formula is made from pasteurized liquid formula that is then spray dried into a powder. It is possible for organisms to be introduced in the final stages of production (Baker, 2002). A microorganism that specifically associated with infant formula is *Cronobacter sakazakii* (previously *Enterobacter sakazakii*). Starting from 1980s, people started to recognize the *E.sakazakii* infections in neonates associated with intrinsic contamination of powdered infant formula (Simmons et al., 1989; Iversen et al., 2004; Drudy et al., 2006). Based on the review published by Bowen and Braden (2006), while rare *C. sakazakii* kills 40% to 80% of infected neonates and has been associated with powdered infant formula. Comparably, Friedemann (2009) published a review on epidemiological studies on *C. sakazakii* infections. It confirmed the high level of lethality of neonatal *Cronobacter* infections with the data of 120 - 150 cases and concluded that contaminated powdered infant formula was the main source of neonatal infections. These outbreaks identified the significance of commercially contaminated formulas and emphasized the need to limit contamination and survival of bacteria in infant formula. Over the past thirty years, pathogenic microbial infections associated with powdered infant formula periodically globally, and primarily involved *C. sakazakii* and *Salmonella enterica* (Usera et al., 1996; Brouard et al., 2007). In many of these cases, low levels of *Salmonella* were detected in the formula. Although the researchers also inferred that only a small proportion of the actual number of *Salmonella* infections in infants were linked to powdered infant formula, it was still a severe problem (Angulo et al., 2008). Managing this problem requires a multidimensional approach including manufacturers, regulators, and caregivers to infants, but eliminating the contamination sources from the production side would

be one of the preferred solutions. The reason that *C. sakazakii* was a particular risk in powdered infant formula was its high thermal resistance. This was particularly true in low moisture foods, such as powdered infant formula and flour, where the effective heat treatment requires longer times and higher temperatures due to increased thermal resistance at low water activities (Syamaladevi et al., 2016). Some early studies evaluated the thermal resistance of different *Cronobacter* strains and other pathogenic microorganisms. In the comparison of D-values at temperatures below 60 °C in reconstituted infant formula, the results indicated *C. sakazakii* was one of the most heat resistant, non-spore forming bacteria among ten typical foodborne bacteria in dairy products with the Z-value at 5.82 °C and D_{60°C} at 2.5 minutes (Nazarowec-White and Farber, 1997).

One way to decrease the thermal resistance was to use some detergent and sanitizer stresses to decrease the thermal resistance of *C. sakazakii* in infant formula (Osaili et al., 2008). In general, the cells exposed to acid, alkaline, chlorine and ethanol stresses had lower D-values in reconstituted infant formula. As the non-thermal technologies advanced in recent decades, researchers also examined the potential of utilizing non-thermal techniques to control *C. sakazakii* in reconstituted infant formula (Pina-Pérez, 2016). However, all of the evaluated non-thermal technologies are still in the early stages of development with the ranking of their potentials as follows: high hydrostatic pressure > gamma irradiation > pulsed electric fields > ultraviolet light. As for preservatives, vanillin, ethyl vanillin, or vanillic acid were evaluated with their bactericidal effect in rehydrated infant formula at 58 °C against *C. sakazakii* (Yemiş et al., 2012). Addition of 20 mM of these three preservatives significantly reduced the thermal resistance of *C. sakazakii* in rehydrated infant formula, but this concentration level was higher

than in other processed foods. These results indicated the need to utilize more effective alternatives to reduce the thermal resistance of *C. sakazakii* in powdered infant formula.

The increased susceptibility of high-risk individuals, including neonates, make effective control of food products that received relatively minimal thermal processing such as pasteurized milk, juices, and ciders have raised concerns about both the adequacy of thermal processes and control of post-pasteurization re-contamination. One of the concerned microorganisms is *Listeria monocytogenes*. During the minimum high-temperature, short-time treatment (71.7 °C, 15 s) required by the U.S. Food and Drug Administration for pasteurizing milk, *L. monocytogenes* cells can still survive through the treatment (Doyle et al., 1987). And it also agreed with the epidemiological studies on the outbreaks linked to *L. monocytogenes* in pasteurized milk (Centers for Disease Control and Prevention (CDC), 2008; Jackson et al., 2011). Likewise, pathogenic microorganisms might also survive in fruit juices, ciders, and concentrates, especially for the cells that are pre-adapted to acid which also increases thermal resistance (Sharma et al., 2005).

Fresh produce and fresh-cut fruits also carry the risk of *C. sakazakii* contamination as they do not go through thermal processing, but some nonthermal processing techniques would also reduce the thermal resistance of the bacteria cells at limited efficacy. The combination of 100 minutes of ultrasound and 200 ppm sodium hypochlorite was experimentally proved to be a potential treatment in leaf vegetable processing as the treatment would introduce more than 1 log of additional *C. sakazakii* inactivation. The results also exhibited the synergistic effect between ultrasound and sodium hypochlorite (Park et al., 2016). UV-C treatment was also proved to be a possible processing technique in fresh-cut fruits: it resulted in more than 2 logs of microbial

reduction than acidic electrolyzed water or neutral electrolyzed water treatment (Santo et al., 2016).

2.2 The Pathogens

This research project studied four pathogenic microorganisms: *C. sakazakii* 607, attenuated *E. coli* O157:H7, *S. enterica* serotype Typhimurium, and *L. monocytogenes*.

- *Cronobacter sakazakii* 607: *Cronobacter* are Gram-negative, rod-shaped, facultative anaerobic, non-spore-forming bacteria belonging to the family *Enterobacteriaceae*. *Cronobacter sakazakii* was originally classified as *Enterobacter cloacae* but was subsequently designated as a separate species (*Enterobacter sakazakii*) on the basis of yellow pigment production, DNA-DNA hybridization, and other phenotypic characteristics (Farmer et al., 1980; 1985). *Cronobacter* was proposed as a new genus by Iverson et al. (2007) to include the organisms formerly classified as *Enterobacter sakazakii*, initially comprising eight different organisms including four named species, one unnamed species, and five named subspecies (Farmer, 2015). Among all of the *Cronobacter* isolates evaluated by Edelson-Mammel et al (2004), *C. sakazakii* 607 had the highest thermal resistance. Detailed heat resistance comparison among *Cronobacter* species was also conducted by other researchers in BHI broth (Breeuwer et al., 2003), in milk and special feeding formula (Osaili et al., 2009).
- *Escherichia coli* O157:H7: *E. coli* is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestinal tract of warm-blooded organisms. It was first identified as a human pathogen in 1982. One of several Shiga toxin-producing serotypes known to cause human illness, the

organism probably evolved through horizontal acquisition of genes for Shiga toxins and other virulence factors (Mead et al., 1998). Although *E. coli* O157:H7 has been not reported to have particularly high heat resistance, several factors can increase its heat resistance, including increased growth temperature, growth phase, reduced water activity and acid adaptation (Kaur et al., 1998; Sharma et al., 2005). The heat resistance of *E. coli* O157:H7 was studied both genetically (Cheville et al., 1996; Mercer et al., 2015) and experimentally in food matrices such as meat and poultry (Ahmed et al., 1995). The *E. coli* O157:H7 strain used in this study is an attenuated version that does not produce Shiga toxin.

- *Salmonella enterica* serotype Typhimurium: *Salmonella enterica* is one of the two species in the genus *Salmonella*. It is a Gram-negative, rod-shaped, motile, facultative anaerobic bacterium. It is subdivided into several subspecies, and almost 3000 unique serovars have been isolated. A number of its serovars are serious human pathogens. Its virulence is due to an outer membrane consisting largely of lipopolysaccharides (LPS) which protect the bacteria from the environment, as well as specific virulence factors that are often associated with specific “pathogenicity islands”. Its heat resistance can be enhanced by pre-adaption at elevated incubation temperatures (Mackey et al., 1986). In epidemiological studies, *Salmonella* cases were often linked to long-term survival in low water activity foods or in low-moisture environments (Archer et al., 1998). The general heat resistance of *S. Typhimurium* can be influenced by a number of environmental factors such as water activity (Sumner et al., 1991), pH (Casadei et al., 2001), and sodium chloride concentration (Mañas et al., 2001).

- *Listeria monocytogenes*: *Listeria* are Gram-positive, non-spore-forming, motile, facultative anaerobic, rod-shape bacterium, with *Listeria monocytogenes* being the most important pathogenic species of the genus. Although *Listeria monocytogenes* is actively motile by means of peritrichous flagella at room temperature (20–25 °C), the organism does not synthesize flagella at body temperatures (37 °C). Invasive infection by *Listeria monocytogenes* causes the disease listeriosis, which is extremely dangerous to pregnant women and neonates, people 65 years and older, and people with compromised immunity. *Listeria monocytogenes* is noted for its ability survive at freezing temperatures and multiply at refrigeration temperatures (Centers for Disease Control and Prevention, 2019), which makes it a great public health concern associated with various ready-to-eat foods, dairy products, and deli meats (Ferreira et al., 2014). Over the years, the general heat resistance of *L. monocytogenes* was also examined and reviewed in detail by multiple research groups (Mackey and Bratchell, 1989; Lemaire et al., 1989; Suarez, 1989; Doyle et al., 2001).

Much of the focus of this study is on *C. sakazakii* survival in different food matrices, so the heat resistance mechanism of *C. sakazakii* will be discussed separately. Previous study showed that heat shock at temperature 42 to 47 °C for 5 to 15 minutes enhances the thermal resistance of *C. sakazakii* at 51 °C (Chang et al., 2009). Heat shock enhances its heat resistance while acid, alkaline, and osmotic stresses decrease the heat resistance of *C. sakazakii* (Arroyo et al., 2011). These results demonstrated the need for innovative ways of decreasing its heat resistance. Studying the heat resistance of highly heat resistant strains, Williams et al., (2004) utilized a proteomics approach designing a biomarker to identify the protein that is unique in the

thermal tolerant strains, demonstrating the potential linkage between specific proteins and elevated thermal resistance. PCR and DNA polymorphism-based analyses verified the gene expression pattern in heat tolerant strains of *C. sakazakii*; heat resistant clones showed higher *infB* expression, which encoded the prokaryotic translation initiation factor (IF2), and it signified the genetic characterization of the heat resistance in *C. sakazakii* (Asakura et al., 2007).

2.3 D-value and Kinetics of Thermal Inactivation

In microbiology, in the context of a sterilization procedure, the D-value (decimal reduction time) is the time (or dose) required, at a given condition (e.g., temperature) or set of conditions, to achieve a 1.0-log reduction, i.e., kill 90% of relevant microorganisms (U.S. Food and Drug Administration, 2014). D-values are temperature-specific for each strain and is commonly used to compare the heat resistance of the microorganisms. For thermal processes, understanding a microorganism's D values allows a processor to measure the amount of microbial inactivation delivered by the process (Institute of Food Technologists, 2000). For a number of thermal processing systems, the total number of microorganisms destroyed by the process can be estimated by incorporating the destruction rate kinetics of the microorganism of concern into the heat transfer model for that system (Institute of Food Technologists, 2000). First-order kinetic model is often assumed. The model assumes that, at a given temperature, the fraction of molecules that will reach the level of energy required for the transformation (e.g., inactivation) to happen is constant. The fraction of molecules that have enough energy to react will increase with temperature (Heldman and Newsome, 2003).

2.4 The “parabens”

2.4.1 Introduction

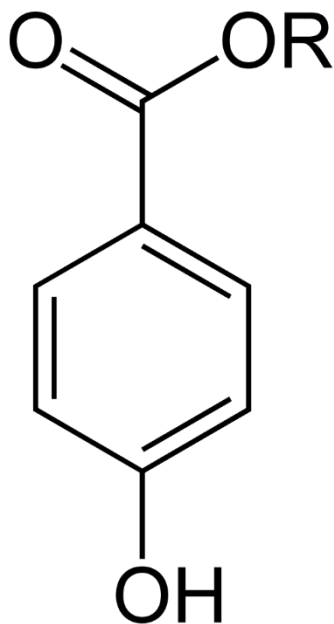


Figure 2-1. Chemical structure of parabens.

Parabens are a series of parahydroxybenzoates (p-hydroxybenzoate), i.e., esters of parahydroxybenzoic acid. They are effective preservatives widely used in cosmetic and pharmaceutical products (Yazar et al., 2011). The basic structure of parabens consists a benzene ring, a changeable ester group, and a hydroxyl group in the para position. (Figure 2-1).

The parabens used commercially are usually industrially synthesized, but they also occur naturally in some sources, including *Microbulbifer* bacterium (Peng et al., 2006; Qué'vrain et al., 2009), and root hairs of the New Zealand yam (Bais et al., 2003). Methyl, ethyl, and propyl parabens along with their

sodium salts are most commonly used parabens for their anti-bacterial and anti-fungal activity. Based on the study of Ruan and Buchanan (2016), the ability of paraben to enhance their inactivation of *C. sakazakii* is positively correlated to the length of side chain, while the water solubility is negatively related to the length of the side chains.

As parabens are widely used in industry, they are also regulated worldwide: Methyl-paraben (21 CFR 184.1490) and propyl-paraben (21 CFR 184.1670) have been granted generally recognized as safe (GRAS) status by the FDA for direct addition to food at levels not to exceed good manufacturing practices: maximum level of 0.1 percent can be added to food. Butyl paraben (<20 ppm) was approved by the FDA for addition to beverages as synthetic flavoring substances and adjuvants (21 CFR 172.515). Heptyl-paraben is permitted by the FDA for direct

addition to fermented malt beverages in amounts not to exceed 12 ppm and in non-carbonated soft drinks and fruit in various foods.

While butyl paraben is the focus of the current study, the literature review discusses parabens in general.

2.4.2 Toxicity

As is commonly the case, when the safety of parabens has been brought to the public's attention, people become concerned about its use in foods, cosmetics, and pharmaceuticals despite the fact that the chemicals are still approved for specific applications. This section will discuss the results of parabens toxicity studies in detail.

Although the toxicity of parabens remains controversial, there is no definite evidence showing that application of parabens as preservatives cause cancer to date. "At this time, we do not have information showing that parabens, as they are used in cosmetics, have an effect on human health" (Food and Drug Administration (FDA), 2018). Starting from the application in industry, people began to get directly exposed to parabens through oral and dermal exposure from personal care products and environmental sources. After the wide application on food products, oral exposure of parabens has increased. Industry estimates of the daily use of cosmetic products that may contain parabens were 17.76 g for adults and 0.378 g for infants (CIR, 2008). And the amount of oral exposure through food is low compared with the dermal exposure through personal care products. Epidemiologic studies are normally conducted through biomonitoring method with measurement of parabens in urine sample. However, biomonitoring study results could not differentiate the exposure route, which was supposed to be one of the major considerations when conducting toxicology studies.

The widely used parabens are usually synthesized in industry, but there are also some naturally occurring ones, which are microbial and plant metabolites. A study showed that marine bacterium biosynthesized pHBA and its alkyl esters at high levels (Peng et al., 2006). The origins of parabens in plant tissue are not fully understood. High levels of paraben production and application also lead to the contamination of surface water. There were several studies conducted on the surface water contamination with parabens worldwide, the results of which indicate that the contamination levels are typically low but highly season-dependent (Błędzka et al., 2014). Besides water, parabens were also detected in soils, sediments and sludge. The likelihood of accumulation of parabens in sediments increased proportionally to the length of side chain (Błędzka et al., 2014). With the usage of personal care products and cleaning agents in household, parabens have been detected in door dust and air, which could lead to humans being exposed inhalation and oral ingestion (Canosa et al., 2007). In general, although there are numerous routes and sources of parabens from the environment, the total dosage of environmental exposure is pretty low compared with personal care products and food. The majority of parabens in environment came from the leakage or direct pollutions from wastewater treatment plants.

Aubert et al. (2012) conducted a study with radioactive parabens of the fate of parabens in rats after oral administration. According to their findings, the overall pharmacokinetics profiles on methyl, propyl and butyl parabens were similar in male and female rats. The majority of an oral dose was eliminated in the urine within the first 168 h. The typical range of the elimination in the urine is more than 90%, with variation due to different parabens and gender. The parabens were normally rapidly eliminated into urine within first 24 h. The typical percentage is around 80% in both male and female. A small amount of orally administrated

parabens was recovered through cage washes of male and female, which takes 6.0% to 14% with variation due to compounds and gender. A tiny portion of parabens, range from 0.9% to 3.0%, were eliminated in the feces. One of the significant findings of this paper is rapidity and efficiency of elimination in rats without differences in gender. The limiting factor in this study was the mixture of methyl, propyl and butyl parabens was a one-time ingestion; subchronic and chronic ingestion exposures are needed.

Some *in vivo* and *in vitro* studies were also conducted to assess the possible carcinogenicity of parabens. Darbre et al., (2003) measured proliferation of human breast cancer cells with benzyl 4-hydroxybenzoate. The cell cultures were incubated with parabens in medium, and the culture medium was changed 3-4 days in the experiment. In other words, the cells were dosed with parabens at a relatively constant concentration over time. Benzyl parabens at 10^{-6} M ($P=0.037$) and 10^{-5} M ($P<0.05$) stimulated cell proliferation, and all the cells were killed at 10^{-4} M. There was no significant antagonism was found at 10^{-7} to 10^{-10} M concentrations. The parabens concentration in this study was relatively high compared to the commercial products. Another issue in this study was that the cells were exposed to parabens continually for 14 days, which could be considered an unrealistic exposure. Considering the fast elimination results from animal study (Aubert et al., 2012), single or multiple doses might be a better experimental design.

Animal studies were also conducted using oral ingestion (Oishi et al., 2004). Methyl parabens and ethyl parabens at 0.1% and 1% concentrations were mixed in the standard diet and delivered to rats, age 25 to 27 days and initially weighing 75.9 ± 2.87 g, for 8 weeks. The control rats was fed with standard diet without any paraben addition. Sperm counts and hormone levels were measured after treatment. No relationship between the sperm counts *in cauda* epididymides

and testis and the application of parabens (Oishi et al., 2004). The concentrations of testosterone, LH and FSH were not be influenced by the addition of parabens (Oishi et al., 2004). The studies discussed above were not in full agreement with prior *in vivo* and *in vitro* studies, which indicated weak estrogenic effects (Prusakiewicz et al., 2007). The results of this specific experiment even did not agree with the authors' previous study, which demonstrated the anti-spermatogenic activity of propyl and butyl parabens (Oishi, 2002). Their previous studies were also an *in vivo* test with rats and mouse, and they compared the toxicity of parabens with alkyl phenols, in which results showed parabens had same or potent anti-spermatogenic activity.

In general, there is no solid evidence confirmed that parabens are carcinogens. On the other hand, there are some studies demonstrated possible weak estrogenic effects of parabens at relatively high doses, although other studies showed there is no estrogenic effect of parabens in animal studies. Based on the current findings, there is no evidence indicating the current exposures of parabens to people are dangerous enough to cause any adverse effects.

2.4.3 Antimicrobial properties and synergism

The antimicrobial mechanisms of parabens are not completely clear at this stage, and this study only focused on their bactericidal properties. There are three proposed modes including disrupting membrane transport processes (Freese et al., 1973), inhibiting synthesis of DNA and RNA, (Nes et al., 1983) or inhibiting some key enzymes, such as ATPases and phosphotransferases (Ma et al., 1996). According to, Bredin et al., (2005), propyl paraben induced potassium release that was related to the porin expression in the bacterial outer membrane in susceptible *E. coli* cells similar to that observed with polymyxin B. They also proposed that this permeabilizing effect is probably related to antibacterial properties of paraben

molecules. The primary advantage of the parabens over benzoic acid is their wider effective pH range (3.0-8.0), whereas the optimum range for benzoic acid is 2.5-4.0. Additionally, the potent antimicrobial activity of parabens enabled use of lower concentrations. One limiting parameter associated with parabens is their low solubility. Adding co-solvent such as ethanol, propylene glycol, and glycerol would be a practical method to increase the aqueous concentrations of parabens above their saturation solubility. Darwish and Bloomfield (1995, 1997) suggested that there was an additive effect between parabens and co-solvent on the integrity of cell membrane permeability causing more cell leakage, however, the antimicrobial effect of the combination was potentially synergistic against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. It was also suggested that the addition of co-solvent reduced partitioning of parabens into the oil phase, which might account for enhanced preservative activity within an emulsion formulation.

Aalto et al., (1953) and Sokol (1952) were the first researchers to determine the minimal inhibitory concentrations of the methyl, ethyl, propyl, and butyl esters using current testing techniques. In general, they found that inhibition of Gram-positive bacteria was proportional to the molecular weight of the ester used. In contrast, inhibition of Gram-negative bacteria was not necessarily related to the ester chain length. On the other hand, Merkl (2010) determined that the minimum inhibitory concentrations (MIC) of parabens against *E. coli*, *Bacillus cereus*, *L. monocytogenes*, *Fusarium culmorum*, and *Saccharomyces cerevisiae*, were all below 5.00 mmol/L. Their results demonstrated that the antimicrobial activity was enhanced as the length of ester chain increased. And these findings generally agreed with the experimental results collected by Ruan and Buchanan (2016). They found the ability to enhance thermal inactivation was positively related with the length of parabens side chain against *C. sakazakii* at mild heating temperatures. According to these results, methyl paraben was the least potent one among the

paraben group. The MIC of methyl paraben was still significantly lower than sodium benzoate against all 10 tested microorganisms except *Aspergillus niger* (Mirsonbol et al., 2014).

There were also a large number of studies on comparing the antimicrobial efficacy of parabens and other preservatives. Moir and Eyles (1992) compared methyl paraben and potassium sorbate, in which the results indicated that the differences between the bacteria were substantial among *L. monocytogenes*, *Pseudomonas putida*, *Yersinia enterocolitica*, and *Aeromonas hydrophila*. The MICs of the two preservatives were similar at pH 5, but at pH 6 the MICs of methyl paraben were well below those of sorbate, except in the case of *A. hydrophila*. The antimicrobial property is not only limited to the parabens but also applies to the potassium salts of methyl, ethyl, propyl, and butyl parabens (Mizuba and Sheikh, 1986). Potassium salts of methyl and ethyl paraben showed lower antimicrobial activity than the corresponding paraben while the potassium salts of propyl and butyl paraben showed higher activity by comparing the MIC against *Aspergillus niger*, *E. cloacae*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa* and *S. aureus*. The advantage of using potassium salts of paraben is that they are more soluble in aqueous systems.

A synergistic effect is one that occurs when two or more agents, factors, or substances that produce an effect greater than the sum of the agent's individual effects. This concept was widely utilized in food processing with various preservatives and foods. For example, organic compound vanillin significantly enhanced *Listeria* inactivation during thermal processing at temperatures from 57 to 61°C (Char et al., 2009). Ding (2017) demonstrated the antimicrobial effect of benzoic acid or propyl paraben treatment combined with UV-A light on *E. coli* O157:H7. Researchers started to estimate the microbial inhibition activity of parabens from 1950s (Aalto et al., 1953; Sokol et al., 1952). In our laboratory (Ruan and Buchanan, 2016), the

addition of low levels of various parabens was found enhance inactivation of a thermally resistant strain, *C. sakazakii* 607, at a mild heating temperature (58 °C). As described later, apple juice was used as a model system in part of the current study. The presence of low levels of benzoic and sorbic acids showed great effect on reducing the heat resistance of *E. coli* O157:H7 at 50 °C (Splittstoesser et al., 1995). A simultaneous application of heat and ultrasonic waves under pressure on the survival of *C. sakazakii* in apple juice was studied by Arroyo et al. (2012). They observed a synergistic effect between 45 °C and 64 °C when combining with ultrasonic waves under pressure against *C. sakazakii* in apple juice. The effectiveness of treatment was further enhanced by the die-off of surviving bacteria during refrigerated storage (up to 96 h at 4°C). Besides apple juice, other pasteurized food such as liquid egg albumen also demonstrated potential synergism (Gurtler and Jin, 2012): although the heat resistance of *Salmonella* generally decreased as the pH rose, it was not practical because of the promoted protein aggregation at high pH. Addition of 500 ppm of propyl paraben to albumen at pH 7.8 reduced the D-value at 56.7 °C more than 11-fold, which demonstrated a significant synergism between heat and parabens.

In the current study, we focused on butyl paraben, and extended our earlier work to determine if the synergy observed extends to other common foodborne pathogens including *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes*, as well as verifying our previous study with *C. sakazakii*. Also, we examined the synergism between heat and butyl paraben in food models: powdered infant formula, non-fat dry milk, and apple juice.

Chapter 3: Project Objectives and Methods

3.1 Project objectives

As discussed in Section 2.4.3, our laboratory has hypothesized that chemicals that disrupt the structure/function of bacterial membranes are likely to have antimicrobial activity and act synergistically to depress bacterial thermal resistance. Research on the effects of butyl-parahydroxy-benzoate (BPB) on foodborne pathogenic bacteria are highly limited in regard to types of food, the effect of cultural conditions on BPB antimicrobial activity, and its effects on thermal resistance. Most studies that have investigated the BPB and other parabens have been proved to limit the growth rates rather than inactivation. Furthermore, most studies were conducted at temperatures that support growth ($< 30\text{ }^{\circ}\text{C}$) and only two prior studies examined the effects of parabens on thermal resistance but only focused on one species. As a means of rectifying this lack of knowledge, the current study was undertaken to examine the potential enhancement of thermal inactivation of four foodborne pathogenic bacteria, *C. sakazakii*, *E. coli* O157:H7, *S. enterica* serotype Typhimurium, and *L. monocytogenes*, during mild heating in a model matrix, Brain Heart Infusion broth (BHI), containing low levels of BPB. The primary objectives in this project were:

1. Using BHI model, examine the effects of various concentration levels of BPB on the effectiveness of mild heat treatments at temperatures $\leq 60\text{ }^{\circ}\text{C}$ for each pathogen.
2. After demonstrating “proof of concept” in BHI model, the second objective was to apply this thermal treatment to “real food systems,” and investigate four parameters that influence BPB antimicrobial ability: product composition, water activity, pH, and lipid solubility.

- a. Powdered infant formula and non-fat dry milk were selected as nutritious dry food models with low moisture/water activity.
- b. Apple juice was selected as a protein-free and fat-free liquid food model with a low pH. This model was also used to investigate the effects of pH on BPB antimicrobial ability and the potential synergistic activity malic acid activity.

3.2 Materials and Methods

3.2.1 Brain and Heart Infusion Broth Studies

Bacterial cultures. *Cronobacter sakazakii* 607 obtained from the Food and Drug Administration, attenuated *Escherichia coli* O157:H7 (ATCC #700728, Manassas, VA), and *Salmonella enterica* serotype Typhimurium and *Listeria monocytogenes* from Dr. Robert Buchanan's lab collection were used throughout the study. Working cultures were sub-cultured monthly on tryptic soy agar (TSA) plates and stored at 4°C.

Butyl-parahydroxy-benzoate. BPB was obtained from MP Biomedicals (99.0+%, catalog No. ICN222907, Solon, OH) was used throughout the study and stored at room temperature.

Submerged coil apparatus. A new submerged coil apparatus (Sherwood Technologies, Model No. Coil-100, Lynnfield, MA) was used in current study, so a thermal resistance study of a known pathogen was conducted to test the consistency of the system. *Cronobacter sakazakii* 607 was used to conduct thermal inactivation study at 58 °C with and without butyl paraben. Following the same methodology that Ruan and Buchanan (2016) used, the control group at 58 °C showed the same thermal resistance and same level of injury. These results confirmed the system consistency and served as basis for studies with the other three pathogens.

Culture preparation and inoculation. Approximately 18-20 h prior to the initiation of an inactivation trial, 10 ml of sterile BHI was inoculated with the target bacterium, incubated at 37 °C, and concentrated by centrifugation at 4800 rpm for 20 min (Beckman GS-15R Centrifuge, Indianapolis, IN). The pellet was re-suspended by mixing with 1 ml of sterile 0.1% peptone water. Prior to the start of a trial, the desired concentration of BPB was solubilized in 70% ethanol and 80 µl of solubilized BPB was added to 19 ml sterile BHI. The trial was then initiated by transferring a 1.0-ml portion of re-suspended culture being transferred to the 19 ml BHI. The inoculated culture was mixed thoroughly, and a 0.4 ml portion retained to as a time-zero sample. The levels of bacteria in the time-zero samples were consistently 10^8 ~ 10^9 CFU/ml.

Thermal inactivation trials. Thermal trials were conducted using a submerged coil apparatus using a slight modification of the procedure of Edelson-Mammel and Buchanan (2004). After programming the instrument with the number of samples to be taken, the appropriate number of 4-ml screw-cap vials, each with 3.6 ml of sterile 0.1% peptone water, were placed on the sample collection carousel. The submerged coil apparatus was pre-equilibrated to the designated temperature and programmed to deliver 400 µl aliquots at designated sample times. A 10-ml portion of the time-zero culture was injected into the submerged coil apparatus, and the program was initialized immediately. After samples were delivered, the vials, which served as 10^{-1} dilutions, were immediately capped and put on ice to halt any further thermal inactivation. The samples were then diluted to 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} using dilution blanks by transferring 0.1 ml of lower dilution sample into 0.9 ml of sterile 0.1% peptone water.

Plating and enumeration. The 10^{-1} , 10^{-3} , and 10^{-5} dilutions were surface plated onto tryptic soy agar (TSA) plates using a spiral plater (Neutec Group Inc., Nr 10003700/113, Farmingdale, NY). The plates were allowed to dry at room temperature for 10 minutes, inverted,

and incubated at 37°C for 20-24 hours. Plates were enumerated using an automatic plate counter (Neutec Group Inc., Nr 10006021/182, Farmingdale, NY), and the numbers were converted to log₁₀ values.

D-values analysis. The collected data were imported into Excel spread sheets and the survivor curves were plotted. D-values of each survivor curve under each treatment condition were calculated by linear regression analysis. In the calculation of slopes, only the points with error bar that did not cross below lower detection limit were included for regression analysis. Student t-test was conducted Excel spread sheets for comparison between 125 ppm heat treatments and 0 ppm heat treatments with significant level at P=0.05.

3.2.2 Powdered Infant Formula and Non-fat Dry Milk Study

Bacteria cultures. *Cronobacter sakazakii* 607 obtained from the Food and Drug Administration was used throughout the study. Working cultures were sub-cultured monthly on tryptic soy agar (TSA) plates and stored at 4°C.

Parabens. Butyl parahydroxy-benzoate (BPB) (99.0+%, catalog No. ICN222907, MP Biomedicals, Solon, OH) and heptyl parahydroxy-benzoate (HPB) (99.0+%, Pfaltz & Bauer, catalog No. 50-749-4189, Waterbury, CT) was used throughout the study and stored at room temperature.

Powdered Infant Formula (PIF) and Non-fat Dry Milk (NFDM). Powdered infant formula purchased from Amazon.com was used throughout the whole study. Non-fat dry milk purchased from Amazon.com was used throughout the whole study.

Baby bottles and petri dish. BPA-free polypropylene baby bottles with nipples purchased from Amazon.com were used throughout the PIF rehydration experiments. Sterile

polystyrene 60 mm-diameter petri dishes were used for sample storage at elevated temperature throughout the non-fat dry milk experiments.

Preparation of culture. Two days prior to the start of a new trial, *C. sakazakii* 607 was inoculated into 10ml of sterile tryptic soy broth (TSB) in a 15ml-centrifuge tube and incubate it at 37 °C for 24 h. The 24-h culture was pelleted by centrifugation at 4800 rpm for 25 min (Beckman GS-15R Centrifuge, Indianapolis, IN). The supernatant was decanted, and the pellet re-suspended by adding 2.0 ml of sterile 0.1% peptone water. A spiral plater (Neutec Group Inc., Nr 10003700/113, Farmingdale, NY) was then used to distribute 1.0 ml of the resuspended culture onto the surface of a TSA plate. After incubation at 37 °C for 24 h, a sterile spreader was used to scrape all the colonies off from plate. The colonies were transferred to a 15ml-centrifuge tube, resuspended in 5ml of sterile 0.1% peptone water, and centrifuged at 4800 rpm for 25 min. After decanting the supernatant, 0.5 ml of sterile 0.1% peptone water was used to re-suspend the pellet.

Inoculation of PIF and NFDM. A 50-g portion of PIF was transferred to a stomacher bag. A micropipetter was used to dropwise inoculate 0.5ml concentrated culture into PIF (or NFDM). The stomacher bag was gently shaken to let PIF (or NFDM) absorb the moisture. The air in the stomacher bag was squeezed out, and then a wooden mallet was used to crush the clumps for 20 to 30 minutes. Then BPB (or HPB) at the desired concentration was solubilized in 70% ethanol, and 80 µl of solubilized BPB (or HPB) was added dropwise to the stomacher bag. The wooden mallet was used again to crush the any remaining clumps for 10 to 20 min. After the obvious clumps have been broken down, some air was allowed into the stomacher bag and then shaken vigorously. The air in the bag was again removed, then sealed, and then allowed to equilibrate at 4 °C for 24 h. After the moisture in PIF (or NFDM) reached equilibrium, an

additional 450 g of PIF (or NFDM) was added to the stomacher bag. After vigorous shaking of the PIF, the air was squeezed out, the bag sealed, and stored at 4 °C for future use. This bag served as BPB treatment group. The control group was prepared in the same manner but received 80 µl of 70% ethanol without BPB (or HPB).

Determination of thermal inactivation kinetics.

- PIF: Three 25-g portions of inoculated PIF was transferred into three baby bottles. Sterile water was preheated to designated temperatures on hot plate, and a 180-ml portion of hot water was quickly poured into baby bottles and followed by 30-second shaking. The 0-time sample was taken immediately after the initial shaking, by transferring a 1.0 ml aliquot into 9.0 ml of sterile 0.1% peptone water. This served as the 10^{-1} dilution. Two samples at 5 and 10 min were subsequently taken from each baby bottle. All samples were then diluted to 10^{-3} , and 10^{-5} , by sequentially transferring 0.1 ml of lower dilution into 9.9 ml of sterile 0.1% peptone water.
- NFDM: Following the same PIF inoculation method, dry NFDM powders were inoculated with *C. sakazakii* 607 and supplemented with BPB. Thirty portions of 3.0 g of inoculated NFDM with designated levels of BPB were distributed into individual petri dishes. Fifteen petri dishes were stored at room temperature (25 °C) and 15 petri dishes were stored in 55 °C. Thirty 3-g portions of inoculated NFDM without addition of BPB were distributed into individual petri dishes. Fifteen petri dishes were stored at room temperature (25 °C), and 15 petri dishes were stored in 55 °C. Thirty 3-g portions of uninoculated NFDM without BPB were distributed into individual petri dishes for the purpose of monitoring background flora. Fifteen petri dishes were stored at room temperature (25 °C), and 15 petri dishes were stored in

55 °C. One petri dish from each of condition was analyzed for microbial load every day. Three grams of NFDM of each portion was separated into three of 1-gram portion, and each 1-gram portion was dissolved in 9 ml of room temperature sterile DI water, which served as 10^{-1} . The samples were then diluted to 10^{-2} , and 10^{-3} using dilution blanks by transferring 0.1 ml of lower dilution sample into 0.9 ml of sterile 0.1% peptone water.

Plating and enumeration. 10^{-1} , 10^{-3} , and 10^{-5} dilutions of PIF and 10^{-1} , 10^{-2} , and 10^{-3} dilutions of NFDM were surface plated onto tryptic soy agar (TSA) plates using a spiral plater (Neutec Group Inc., Nr 10003700/113, Farmingdale, NY). The plates were dried at room temperature for 10 minutes, and then inverted and incubated at 37 °C for 20-24 h. Plates were enumerated using an automatic plate counter (Neutec Group Inc., Nr 10006021/182, Farmingdale, NY), and the counts were converted to \log_{10} values.

Data analysis. The collected data were imported into Excel spread sheets and the survivor curves were plotted and compared.

3.2.3 Apple Juice Study

Bacteria cultures. *Cronobacter sakazakii* 607 was used throughout the study. Working cultures were sub-cultured monthly on tryptic soy agar (TSA) plates and stored at 4°C.

Butyl parahydroxy-benzoate. BPB obtained from MP Biomedicals (99.0+%, catalog No. ICN222907, Solon, OH) was used throughout the study and stored at room temperature.

Malic acid. D-Malic acid obtained from Sigma-Aldrich ($\geq 97.0\%$, catalog No. MFCD00004245, St. Louis, MO) was used throughout the study and stored at room temperature.

Apple juice. Shelf stable pasteurized 100% apple juice from concentrate (from Tropicana) purchased from Amazon.com was used throughout the apple juice related experimentation. The juice was stored at room temperature prior to opening to conduct an experiment trial.

Apple juice preparation and inoculation. Approximately 18-20 h before initiation of an experimental trial, 10 ml of BHI was inoculated with target strain, and incubated at 37 °C. The culture was pelleted by centrifugation at 4800 rpm for 25 min (Beckman GS-15R Centrifuge, Indianapolis, IN) and then re-suspended in 1.0 ml of sterile 0.1% peptone water.

The pH of the apple juice to be used was recorded using a pH meter with a combination electrode (Fisherbrand AE150 pH Benchtop Meter, Waltham, MA). In trials examining the effect of pH on BPB enhancement of thermal processing, the apple juice was adjusted to the desired pH using 10 N HCl and/or 1 N NaOH. Prior to a start of trial, BPB with the desired concentration was dissolved in 70% ethanol, and 80 µl of the solubilized BPB was then added to 19 ml apple juice at designated pH value. A 1.0-ml portion of re-suspended bacterial culture was transferred to the 19 ml apple juice, agitated thoroughly, and immediately used to initiate a thermal survival trial. As before, a portion of the inoculated was retained as a time-zero sample. The initial levels of bacteria in the time-zero samples were approximately 10^8 ~ 10^9 CFU/ml.

Determination of thermal inactivation kinetics. Thermal trials were conducted using the submerged coil apparatus. As described earlier, after programing the instrument with the number of samples to be taken, the appropriate number of 4-ml screw-cap vials, each with 3.6 ml of sterile 0.1% peptone water were placed on the sample collection carousel. The submerged coil apparatus was pre-equilibrated to the designated temperature and programmed to deliver 400 µl aliquots at designated sample times. A 10-ml portion of the inoculated time-zero sample was

loaded into a syringe, injected into the submerged coil apparatus, and the program was initialized immediately after injection. At the designated times, the 400 µl samples were dispensed into vials, producing 10^{-1} dilutions. The vials were immediately capped and put on ice to halt any further thermal inactivation. The samples were then diluted to 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} using dilution blanks by transferring 0.1 ml of lower dilution sample into 0.9 ml of sterile 0.1% peptone water.

Plating and enumeration. The 10^{-1} , 10^{-3} , and 10^{-5} dilutions were surface plated onto tryptic soy agar (TSA) plates using a spiral plater (Neutec Group Inc., Nr 10003700/113, Farmingdale, NY). The plates were allowed to dry at room temperature for 10 minutes, inverted, and incubated at 37°C for 20-24 h. The plates were enumerated using an automatic plate counter (Neutec Group Inc., Nr 10006021/182, Farmingdale, NY), and the counts converted to \log_{10} values.

Data analysis. The collected data were imported into Excel spread sheets and the survivor curves were plotted, D-values calculated. Some of the treatments induced extremely rapid inactivation rates, in which the calculation of D-values was estimated with some degree of uncertainty. In this case, the estimated time to achieve a 6-log reduction was calculated and compared for each treatment. Student t-test was conducted Excel spread sheets for comparison between 125 ppm heat treatments and 0 ppm heat treatments with significant level at $P=0.05$.

Chapter 4: Collection and Analysis of Inactivation Data

4.1 Impact of Butyl parahydroxy-benzoate on thermal resistance in a model system

4.1.1 Preliminary study to select thermal treatment temperatures

Preliminary tests confirmed that the thermal resistance of *C. sakazakii* 607 as measured using a submerged coil apparatus was consistent with previous studies (Mammel-Edelson and Buchanan, 2004; Ruan and Buchanan, 2016), and was equally effective for characterizing the thermal resistance of the other three microorganisms evaluated in this study. The experimental design that was selected to assess the ability of BPB to enhance thermal inactivation was based on selecting a thermal process temperature, which in the absence of BPB reduced the population of the target bacterium by 1 -2 log cycles in 900 sec (15 min). Accordingly, a preliminary study was performed to assess the thermal resistance of *S. enterica* serotype Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* at 54 °C to 60 °C. The estimated D-values for these three bacteria are presented in Table 4-1. The survivor curves for *S. enterica* and *E. coli* were reasonably log-linear, and single D-values could be estimated. *Listeria monocytogenes* displayed biphasic inactivation kinetics at the lower heating temperatures potentially suggesting a more heat resistant subpopulation. Two D-values were calculated in this instance, D₁ for the more heat sensitive population and D₂ for the more heat resistant subpopulation. At 56°C, *L. monocytogenes* survivor curve approached log-linear kinetics; however, the reductions exceeded the goal of a 1 to 2 log reduction for the controls without parabens. Prior studies (Ruan and Buchanan, 2016) had demonstrated that thermal processing at 58 °C yielded a 1 to 2 log reduction of *C. sakazakii* during a 900 sec treatment. This was confirmed in the preliminary study. Based on these results, the heating temperatures subsequently used to assess the effects of

BPB were 58°C for *C. sakazakii*, 57 °C for *Escherichia coli* O157:H7, 55 °C for *Salmonella enterica*, and 54 °C for *Listeria monocytogenes*. The survivor curves at these temperatures are depicted in Figure 4-1.

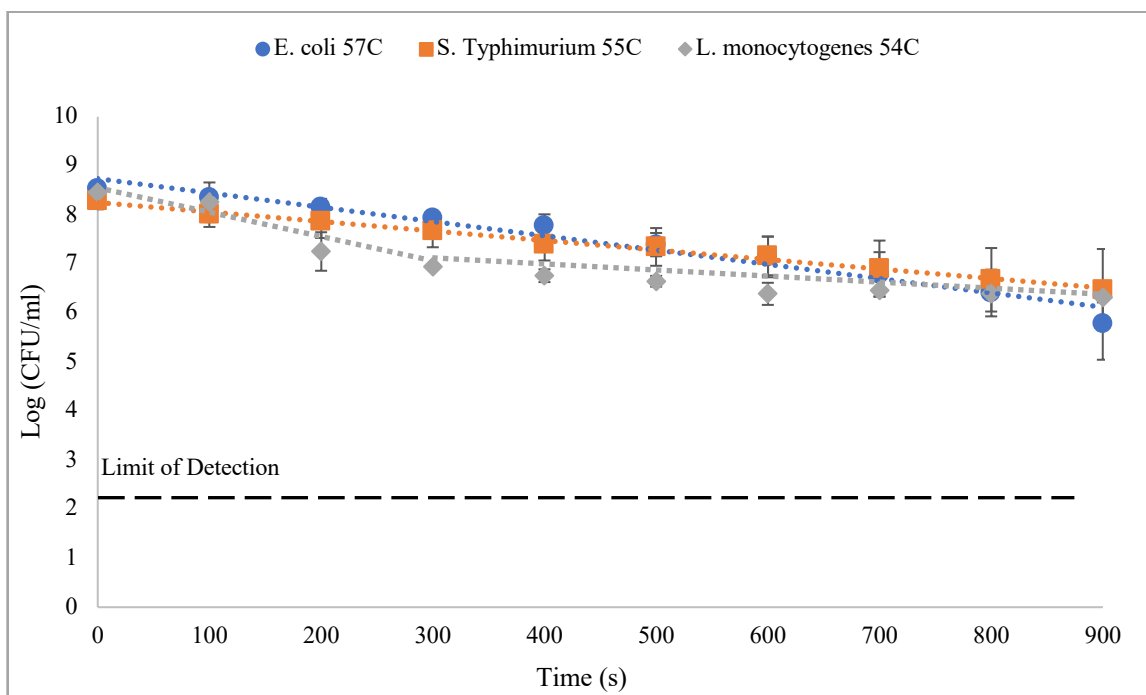
Table 4-1: D-values of the four microorganisms heated at temperatures between 54° and 60°C in the absence of butyl parahydroxy-benzoate.

| Temperature (°C) | D-values (s) | | | | |
|---------------------|------------------------------|---------------------------------|----------------------------|-------------------------------|----------------|
| | <i>Cronobacter sakazakii</i> | <i>Escherichia coli</i> O157:H7 | <i>Salmonella enterica</i> | <i>Listeria monocytogenes</i> | |
| | | | | D ₁ | D ₂ |
| 54 | --- ^a | --- | --- | 179.8 | 1000.0 |
| 55 | --- | --- | 571.5 | 182.7 | 2777.8 |
| 56 | --- | 909.1 | 169.5 | 149.2 | b |
| 57 | --- | 365.7 | 151.5 | --- | --- |
| 58 | 289.3 | 149.2 | --- | --- | --- |
| 59 | --- | --- | 158.7 | --- | --- |
| 60 | --- | 138.9 | 147.1 | --- | --- |

^a Dash line indicates that assay was not performed at this temperature.

^b Inactivation kinetics approached linearity at this temperature so no D₂ was calculated.

Figure 4-1: Survivor curves of control group of *E. coli* O157:H7 (57 °C), *S. Typhimurium* (55 °C), and *L. monocytogenes* (54 °C)



4.1.2 Effect of Butyl parahydroxy-benzoate on Thermal Inactivation of Foodborne Pathogenic Bacteria in Model System

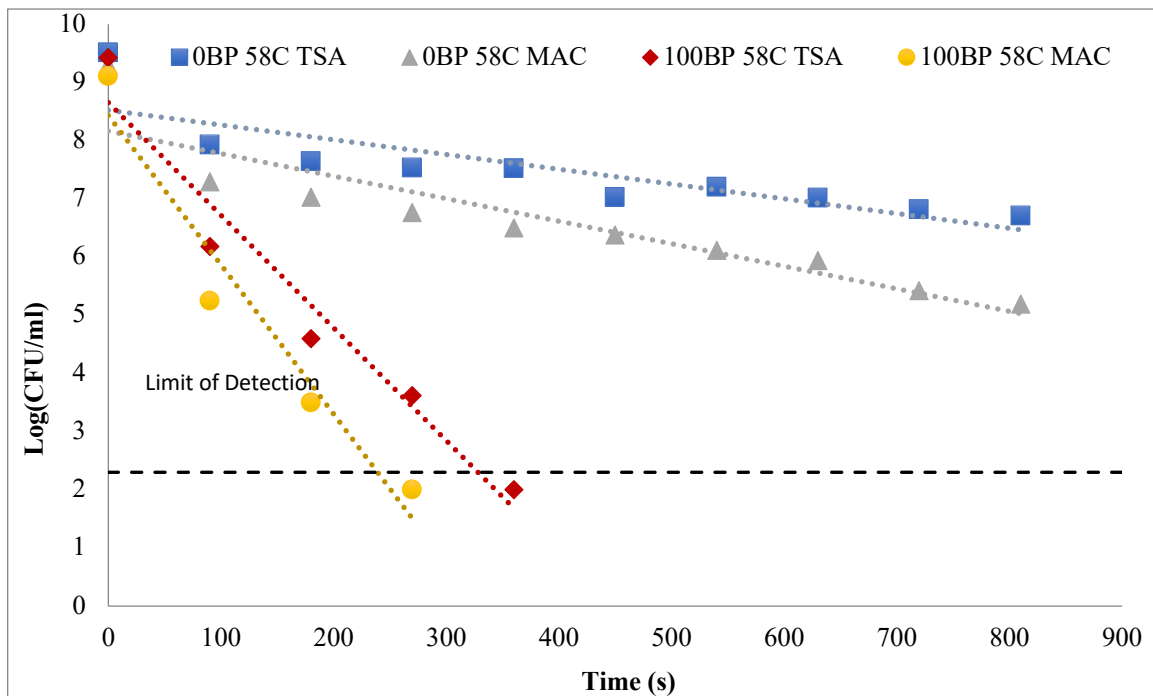
The thermal resistance of *C. sakazakii*, *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* were evaluated at the selected mild heating temperatures (see section 4.1.1) in BHI containing low levels BPB to determine if the compound enhanced the thermal inactivation.

• 4.1.2.1 *Cronobacter sakazakii* 607

Cronobacter sakazakii heated at 58 °C had a D-value at 289.3 sec (Table 4-1) in the absence of BPB. Initial characterization of the effect of BPB on thermal inactivation used a concentration 100 ppm BPB using a dual media system where samples were plated on tryptic soy agar (TSA) and MacConkey Agar (MAC). The former was used to enumerate both injured and non-injured cells while the latter only supported the growth of non-injured cells, allowing

assessment of non-lethal injury (Fig.4-2). The zero-time samples showed no significant degree of injury, but relatively rapid ~1.5 log reduction by 100 sec, followed by a log-linear inactivation over the heat treatment. The inclusion of 100 ppm BPB enhanced the thermal inactivation of *C. sakazakii*, with a 6 – 7 log reduction within 400 sec, compared to the ~2-log reduction in the controls. The degree of injury increased with heating duration in both the controls and the BPB containing cultures. These results are consistent with those observed by Ruan and Buchanan (2016).

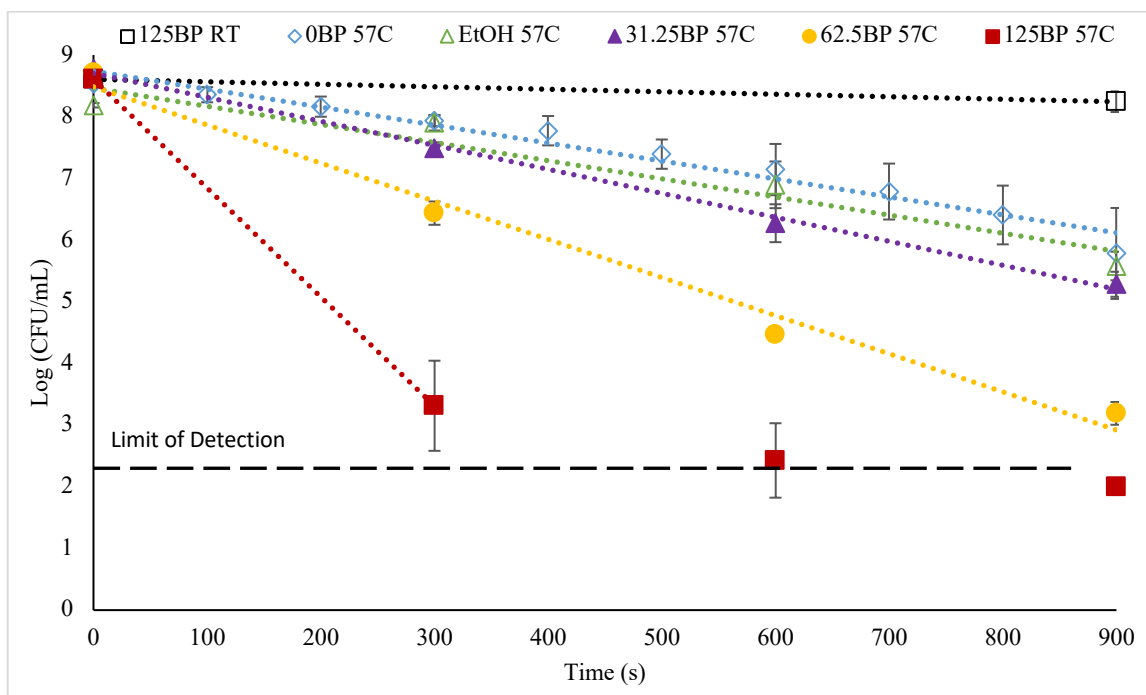
Figure 4-2: Survivor curves of *C. sakazakii* heated at 58°C in BHI with and without 100 ppm BPB and then plated on Tryptic Soy Agar and MacConkey Agar to assess the degree of injury



- 4.1.2.2 *Escherichia coli* O157:H7

The temperature selected for attenuated *E. coli* O157:H7 was 57 °C with the D-value at 365.7 ± 95.0 seconds (see Table 4-1 and Fig. 4-1). The inclusion of BPB resulted in a concentration dependent enhancement of thermal inactivation (Fig. 4-3), with the highest concentration producing a > 6-log reduction after approximately 400 sec. The synergistic nature of this effect was assessed by including a 125 ppm room temperature (RT) control which indicated that at this concentration, BPB had little if any effect at that temperature over the course of 900 sec. Since a low level of 70% ethanol was used to help solubilize the BPB, an ethanol control was performed and indicated that the ethanol did not affect the thermal inactivation kinetics. The D-values observed are provided in Table 4-2. The survivor curves (Figure 4-3) and calculated D-values (Table 4-2) indicated that the addition of ethanol did not have significant effect compared to the control group with no addition of ethanol or butyl parabens. On the other hand, with the addition of BPB, the microbial inactivation at 57°C was significantly enhanced even with the lowest concentration, which was 31.25 ppm, with the D-value at 257.4 ± 16.2 seconds ($P < 0.05$). And the highest concentration 125ppm decreased the D-value to 162.2 ± 3.3 seconds. There was no noticeable antimicrobial effect at room temperature even with inclusion of 125 ppm BPB, which was the highest concentration tested. The combination of the results indicated BPB.

Figure 4-3: Survivor curves of attenuated *E. coli* O157:H7 at 57°C as affected by BPB concentrations



* All of points represented three replicates.

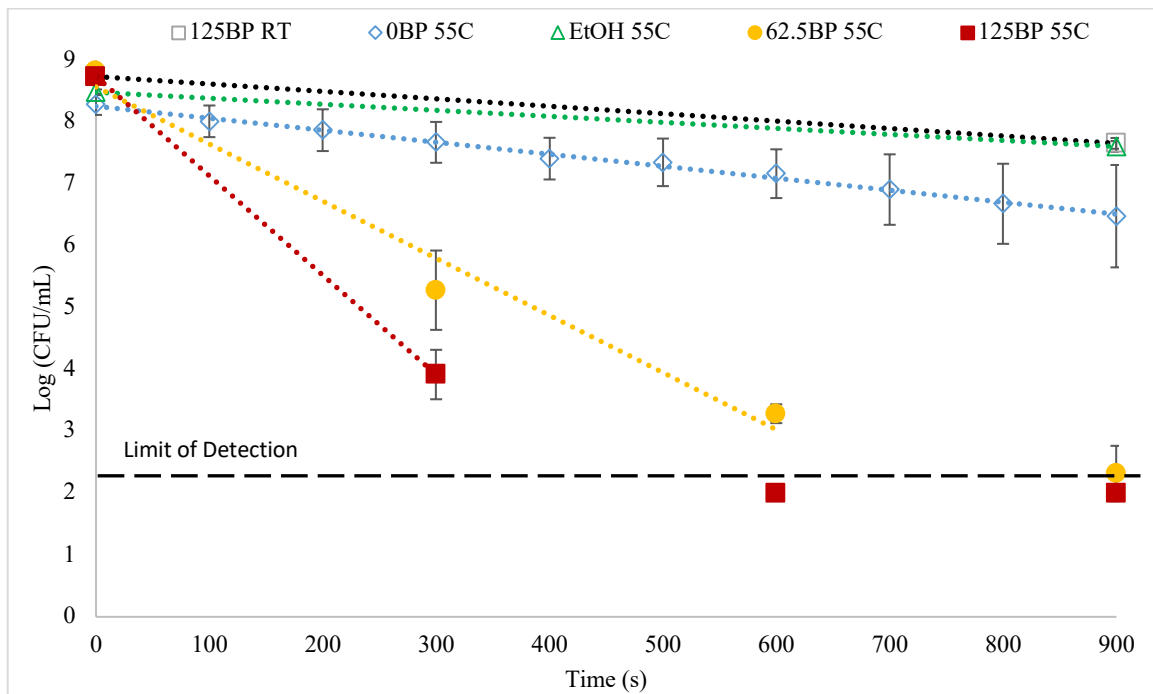
Table 4-2: D-values for attenuated *E. coli* O157:H7 at 57°C as affected by BPB concentrations.

| Treatment | D-value (s) |
|----------------------|----------------|
| 125 ppm BPB + RT | 2599.8 ± 609.7 |
| 0 BP + 57°C | 365.7 ± 95.0 |
| EtOH + 57°C | 339.9 ± 31.9 |
| 31.25 ppm BPB + 57°C | 257.4 ± 16.2 |
| 62.5 ppm BPB + 57°C | 162.2 ± 3.3 |
| 125 ppm BPB + 57°C | 71.4 ± 27.2 |

• 4.1.2.3 *Salmonella enterica* serotype Typhimurium

The temperature selected for *S. Typhimurium* was 55 °C with the D-value at 571.5 ± 168.6 seconds. Two concentrations of BPB were tested: 62.5 ppm and 125 ppm. At 55 °C, the D-values of *S. Typhimurium* with 62.5 ppm and 125 ppm BPB were 108.3 ± 2.4 seconds and 62.8 ± 5.5 seconds, respectively (Table 4-3 and Fig. 4-4). Low concentrations of BPB significantly reduced the D-values of *S. Typhimurium* at 55 °C in a concentration dependent manner ($P < 0.05$). At room temperature, 125 ppm BPB also showed slight antimicrobial activity with D-value at 971.3 ± 400.6 seconds, which was not observed in other three microorganisms. The ethanol control for *S. Typhimurium* at 55 °C appeared to have a protective effect compared to the 0 ppm BPB control trial; however, the slopes of the survivor curve (and thus their D-values) were not significantly different ($P > 0.05$).

Figure 4-4: Survivor curves of *S. Typhimurium* at 55 °C as affected by BPB concentrations



* All of points represented three replicates.

Table 4-3: D-values of *S. Typhimurium* 55 °C as affected by BPB concentrations

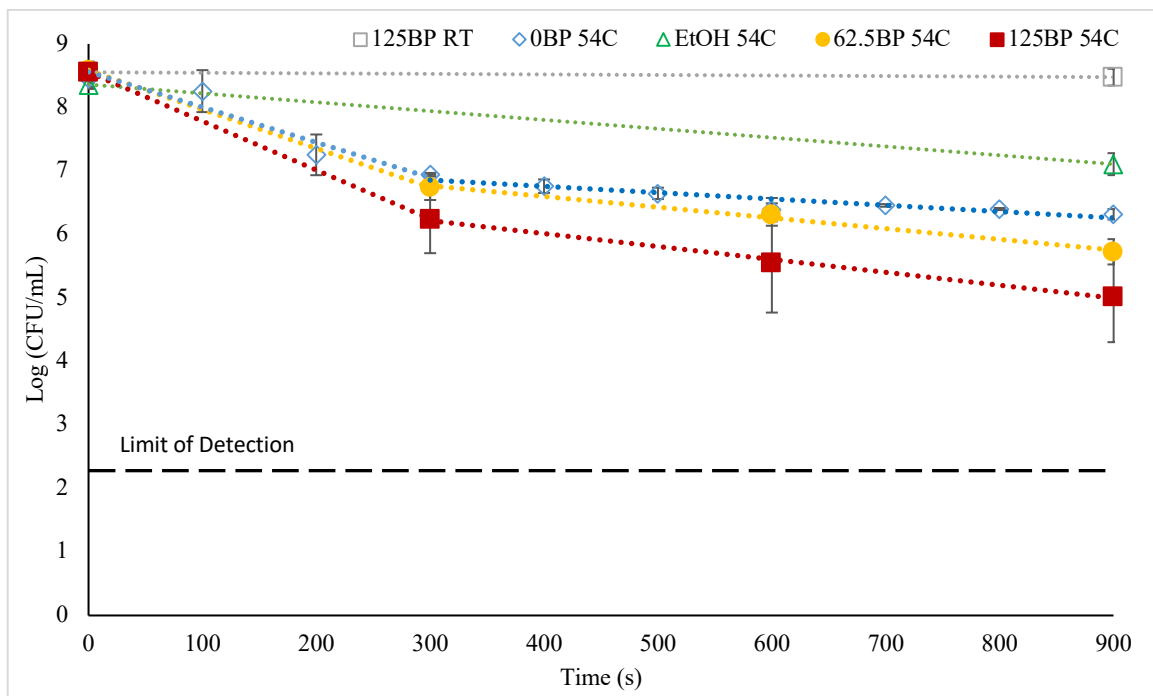
| Treatment | D-value (s) |
|----------------------|--------------------|
| 125BP + RT | 971.3 ± 400.6 |
| 0BP + 55°C | 571.5 ± 168.6 |
| EtOH + 55°C | 1047.6 ± 141.5 |
| 62.5BP + 55°C | 108.3 ± 2.4 |
| 125BP + 55°C | 62.8 ± 5.5 |

- **4.1.2.4 *Listeria monocytogenes***

Listeria monocytogenes was the only Gram-positive organism evaluated in this study. It displayed a distinctly different pattern of survivor curves (Fig. 4-1 and 4-5). The preliminary studies indicated a two-phase inactivation process at 54 and 55 °C. The final inactivation temperature selected for *L. monocytogenes* was 54 °C, at which the microbial population decreased about 2 logs during the 900-sec heating trials, but clearly displayed two-phase inactivation kinetics. As a result, individual D-values were calculated for each inactivation kinetic phase. Comparison of the 0 ppm BPB controls against the ethanol control suggested that the ethanol may have a small protective effect during the 54 °C heating, which could worth to perform some future experiments and statistical analysis to confirm this effect. There was no observable inactivation at room temperature with 125 ppm BPB, which is in general agreement with the results observed with the other three bacteria (Fig 4-5 and Table 4-5). Two BPB concentration levels were tested with *L. monocytogenes*: 62.5 and 125 ppm. In the first phase of

the heat treatment (0 to 300 sec), there was no significant difference in the slopes of the 0 ppm BPB control group at 54 °C and the 62.5 and 125 ppm BPB treatment groups ($P>0.05$). From 300 to 900 seconds, there was a concentration dependent BPB enhancement of the thermal inactivation. However, the enhancement was substantially less than that noted with the other microorganisms as reflected in the phase 2 D-values (Table 4-4 and 4-5). As a result, 125 ppm BPB group only achieved an approximate 4-log reduction during the 900 sec heating cycle, whereas the other three bacteria had > 6 -log reductions with the same level of BPB.

Figure 4-5: Survivor curves of *L. monocytogenes* at 54°C as affected by BPB concentrations



* All of points represent the mean of three replicates.

Table 4-4: D-values of *L. monocytogenes* 54°C as affected by BPB concentrations

| Treatment | D-value (sec) | |
|----------------------|-----------------|----------------|
| | D ₁ | D ₂ |
| 125BP + RT | 5416.7 ± 3280.8 | - |
| 0BP + 54°C | 179.8 ± 5.4 | 1000 ± 0 |
| EtOH + 54°C | 736.0 ± 111.4 | - |
| 62.5BP + 54°C | 164.8 ± 19.4 | 589.6 ± 28.4 |
| 125BP + 54°C | 147.1 ± 56.7 | 508.3 ± 92.0 |

Table 4-5: D-values of *C. sakazakii* 607, *E.coli* 0157:H7, *S. Typhimurium*, and *L. monocytogenes* as affected by BPB concentrations

| D-values (s) | | RT + 125 ppm BP | Heat + 0 ppm BP | Heat + EtOH | Heat + 31.25 ppm BP | Heat + 62.5 ppm BP | Heat + 125 ppm BP |
|--|-----------|-----------------------|-----------------------|-------------------|---------------------------|--------------------------|-------------------------|
| <i>C. Sakazakii</i> 607 (58 °C) | | N/A | 400.0* | N/A | 218.9* | 162.0* | 86.5* |
| <i>E. Coli</i> O157:H7 (57 °C) | | 2599.8 ± 609.7 | 365.7 ± 95.0 | 339.9 ± 31.9 | 257.4 ± 16.2 | 162.2 ± 3.3 | 71.4 ± 27.2 |
| <i>S. Typhimurium</i> (55 °C) | | 971.3 ± 400.6 | 571.5 ± 168.6 | 1047.6 ± 141.5 | N/A | 108.3 ± 2.4 | 62.8 ± 5.5 |
| <i>L. monocytogenes</i> (54 °C) | D1 | 5416.7 ± 3280.8 | 179.8 ± 5.4 | 736.0 ± 111.4 | N/A | 164.8 ± 19.4 | 147.1 ± 56.7 |
| | D2 | N/A | 1000 ± 0 | N/A | N/A | 589.6 ± 28.4 | 508.3 ± 92.0 |

The numbers represent mean of three replicates ± standard deviation.

*Lack of enough replicates to calculate standard deviation, however, values are consistent with results of Ruan and Buchanan (2016).

4.2 PIF and NFDM studies

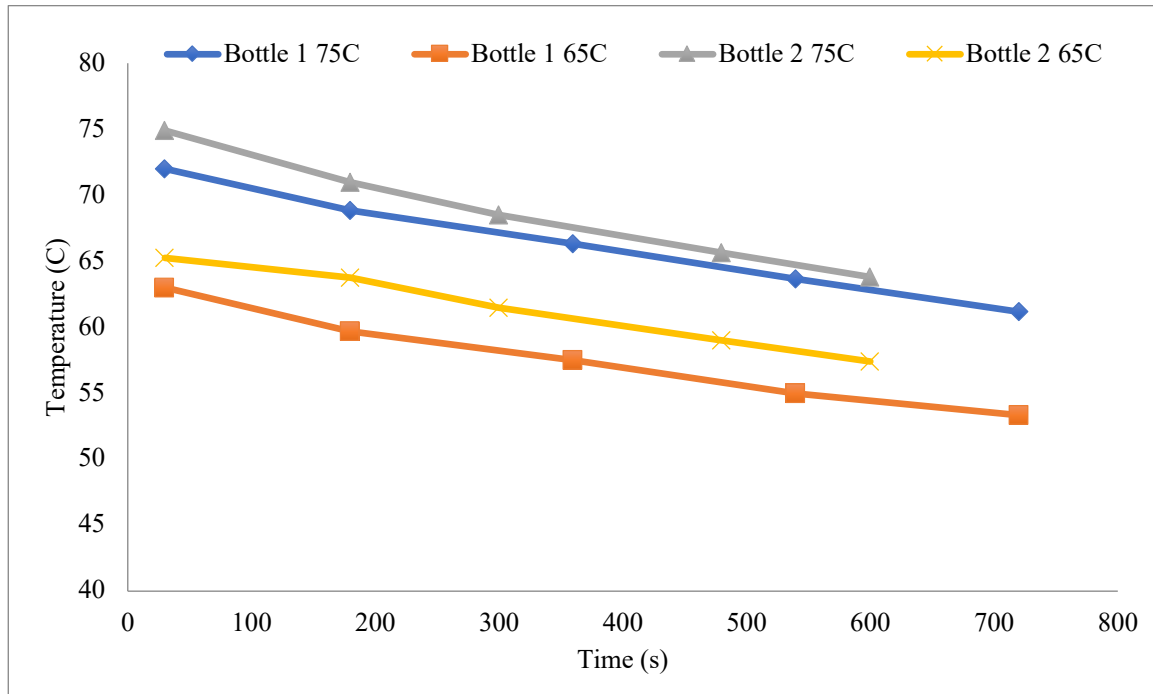
4.2.1 PIF studies

The PIF study was conducted by simulating the rehydration process, in which BPB was added in inoculated PIF during storage and thermal processing was applied by adding hot water at designated temperatures.

- **4.2.1.1 Preliminary studies on the rehydration parameters**

The experiments of PIF were mainly conducted in baby bottles by mixing hot water with one serving size of PIF. The microbial load was monitored for first 10 minutes, so the temperature drop was also monitored during this period. The experiments indicated that the initial PIF temperature did not affect water temperature drop in baby bottles: mixing 65 °C water with 4 °C PIF showed same temperature change compared with mixing 65 °C water with room temperature PIF. (Data not shown.) In PIF studies, the preliminary experiments demonstrated the temperature drop in baby bottles (Figure 4-6). In general, the water temperature decreased at a rate 1 °C per minute. The four curves represented four different trials, each containing three replicates. Two trials started with 75 °C water and the other two trials started with 65 °C water. The timer initiated at the moment the water was added in, and the first temperature record was taken at 30 seconds, which was also the first microbial sampling point in the following experiments.

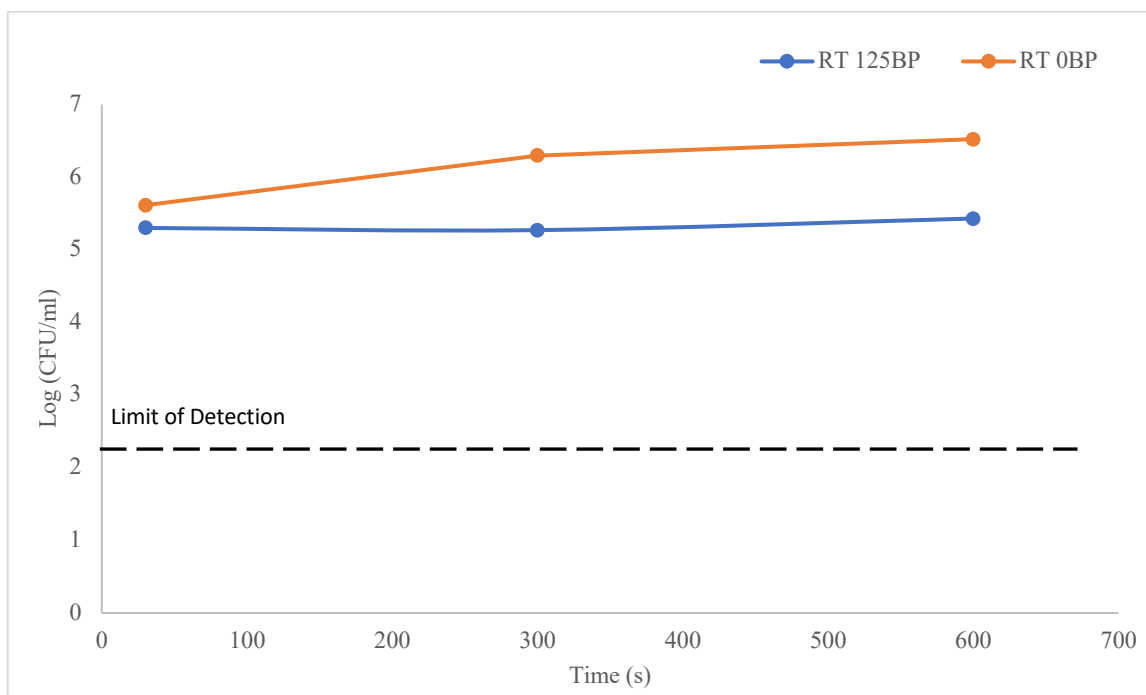
Figure 4-6: Water temperature drop in baby bottles.



- 4.2.1.2 Rehydration of inoculated PIF in baby bottles

Preliminary trials compared the effect of incorporating BPB into PIF at a level that would be equal to 125 ppm when the PIF was rehydrated. When PIF with and without BPB and inoculated with *C. sakazakii* was rehydrated with room temperature water, no significant antimicrobial effect was observed ($P>0.05$) (Fig. 4-7).

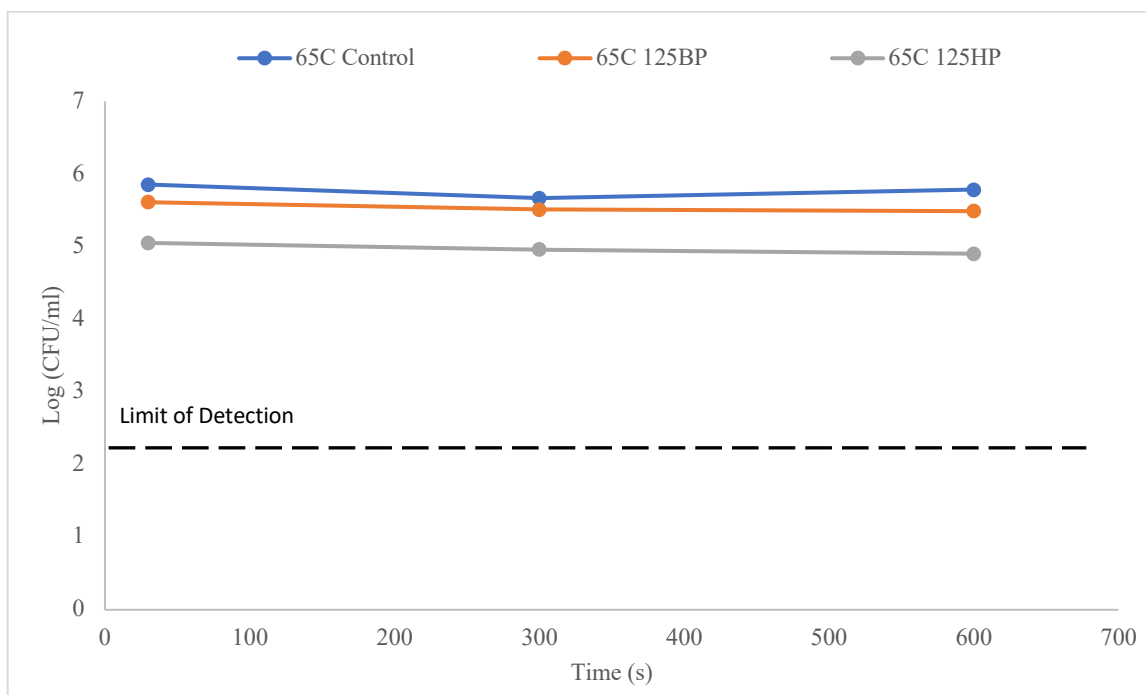
Figure 4-7: Survivor curves of *C. sakazakii* in PIF with 125 ppm BPB and without BPB rehydrated with room temperature water.



* All of points represented two replicates.

The initial trials of PIF hot water rehydration with control group started with water temperatures at 70 °C and 80 °C. Both temperatures enabled “instantaneous” inactivation of *C. sakazakii* to levels below lower detection limit even when the first sampling times was as short as 30 sec (data not shown). These results generally agreed with previous studies (Edelson-Mammel and Buchanan, 2004). When the PIF was rehydrated with 65 °C. (Fig. 4-8), the results indicated that water temperatures ≤ 65 °C did not inactivate *C. sakazakii* with or without BPB or HPB during the 10 min exposure. The microbial population difference between BPB group and HPB group was mainly resulted from the batch difference.

Figure 4-8: Survivor curves of *C. sakazakii* in PIF containing no parabens, 125 ppm BPB, and 125 ppm HPB upon rehydration with 65 °C water.



* All of points represented average of two replicates.

- 4.2.1.3 Potential inhibitory components of PIF that quench the antimicrobial effect of parabens

Based on the preliminary experiments on temperature drop that occurs while rehydrating PIF in baby bottles (see section 4.2.1.1), the water temperature drops from 65 °C to nearly 55 °C after 10 min, a temperature that is still potentially inactivate *C. sakazakii*. However, this was inconsistent with the rehydration trials (Fig 4-8), suggesting that the PIF was protective in relation to thermal inactivation. Furthermore, the lack of enhancement due to inclusion of parabens during mild heating temperatures in baby bottles suggested a potential inhibitory phenomenon due to some component of PIF that interfered with enhancement of thermal inactivation by parabens. As the side chain of parabens become longer, they become increasingly insoluble in water but soluble in fat content. Thus, one possibility was that in a PIF emulsion after rehydration, the microorganisms would be largely in the aqueous phase and would not

come in contact with the parabens which would partition into the non-aqueous phase.

Alternatively, the composition of PIF is quite complex and one or more of the ingredients could be binding or inactivating the BPB or HPB.

To evaluate this potential quenching phenomenon, two sets of experiments were designed and executed:

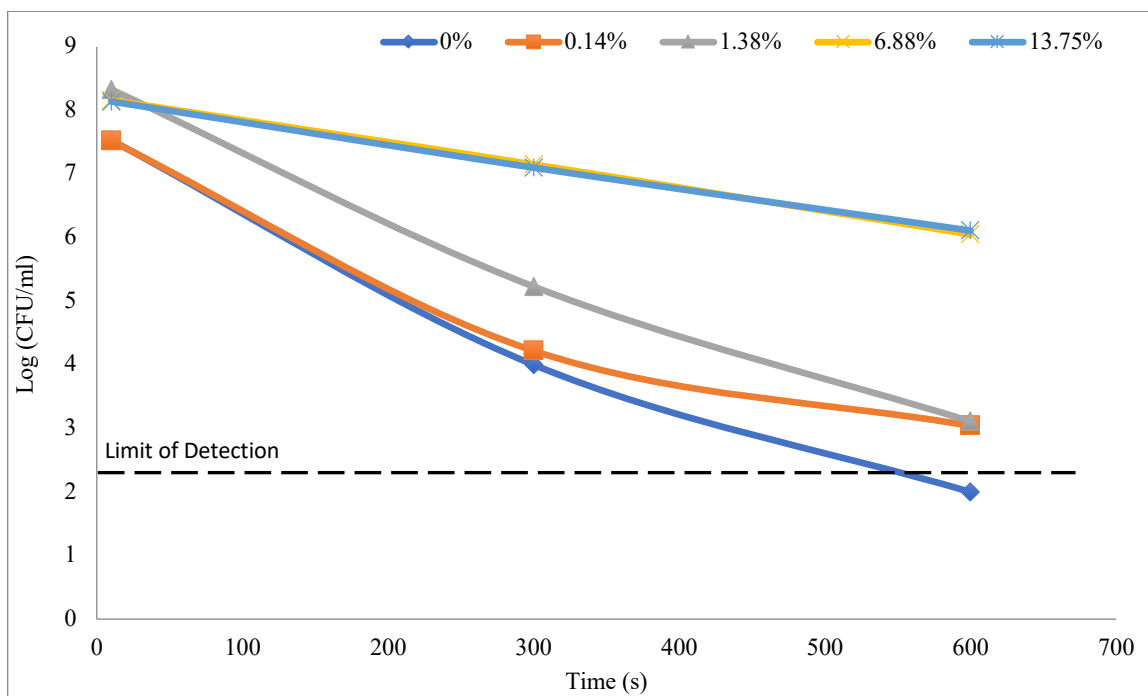
- Substitute PIF with NFDM to avoid BPB partitioning into a lipid phase and use in conjunction with BHI containing different levels of NFDM as the experimental matrix in thermal treatment trials using the submerged coil apparatus.
- Isolate the major content of PIF and conduct fluorescence testing to evaluate potential binding between PIF and BPB.

4.2.1.3.1 Dose response relationship between NFDM content and degree of thermal

inactivation. This experiment was conducted using the protocol employed in the submerged coil model system studies (see section 3.2.1), substituting varied levels of NFDM in place of PIF. At full strength, 25 g of NFDM were dissolved in 180 ml of water (13.75 % by weight which equivalent used to level of rehydrated PIF), followed addition of 125 ppm BPB, inoculation with *C. sakazakii* and heating at 58 °C. In addition to the full-strength level of NFDM, NFDM was added at levels 0%, 1%, 10%, and 50% of full concentration level, so the final percentage was 0% (regular BHI), 0.14%, 1.38%, 6.88%, and 13.75% of NFDM in final liquid mixture. The survivor curves of *C. sakazakii* at 58 °C indicated a clear dose-response relationship (Fig. 4-9). At 13.75% and 6.88% of NFDM, there was no enhancement of thermal inactivation by inclusion of 125 ppm BPB, showed complete inhibition of BPB enhancement of microbial inactivation, i.e., there was approximately a 2-log inactivation after 10 minutes, which is roughly equivalent to

the degree of inactivation observed with *C. sakazakii* in BHI broth without paraben at 58 °C (see Fig. 4-2). At 1.38% NFDM, some degree of enhanced thermal inactivation was apparent, and at 0.14% and 0.00% NFDM levels, the enhancement of thermal inactivation was roughly equivalent to what was observed with 125 ppm BPB in the BHI model system studies (Fig 4.2). The results in Figure 4-9 represented two runs of the experiment with duplicate plating, which was not powerful enough to perform statistical analysis but clear enough to show the general trends of the results.

Figure 4-9: Survivor curves of *C. sakazakii* in heated at 58 °C in solutions containing different levels of NFDM and containing 125 ppm BPB.

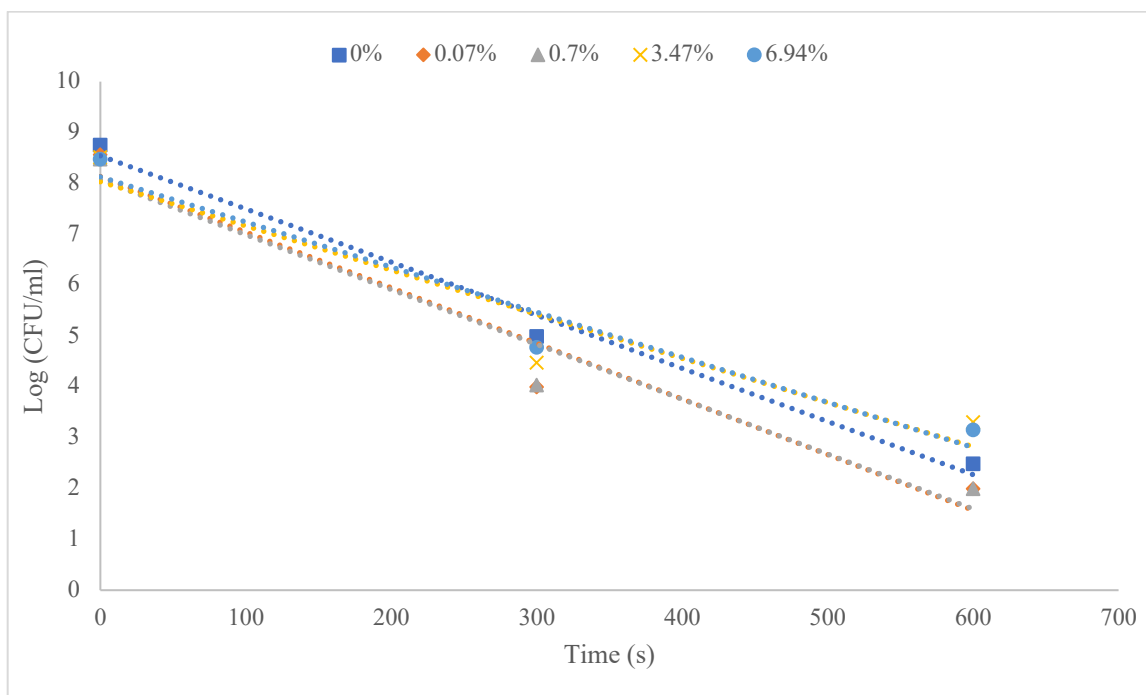


* All of points represented two replicates.

4.2.1.3.2 Dose response relationship between lactose content and enhancement of thermal inactivation by BPB. Approximately 50% of PIF content is lactose, so a similar microbial

inactivation kinetic study was conducted with different levels of lactose to determine if the sugar depressed the enhancement of thermal inactivation by BPB. The rehydrated full serving size of PIF concentration was 0.14 gram/ml. Based on this number, we calculated the percentage of lactose in the rehydrated full serving portion of PIF, which was 6.94% by weight. Using 6.94% as a baseline lactose level, 3.47%, 0.69%, 0.07% and 0% (regular BHI) lactose were used to conduct *C. sakazakii* inactivation kinetic study at 58 °C. The survivor curves of these five lactose concentrations are summarized in Fig. 4-10. All five groups achieved 5 to 6 log reductions in 600 seconds without noticeable differences in slopes between 0 to 300 seconds. There were no concentration dependent differences in the inactivation, and the degree of inactivation was equivalent to that observed in the model system study (Fig 4.2). The results in Figure 4-10 represented one run of experiment with duplicate plating, which was not enough to perform statistical analysis but clear enough to show the general trends of the results.

Figure 4-10: Survivor curves of *C. sakazakii* in BHI containing 125 ppm BPB and different concentrations of lactose and then heated at 58 °C.



* All of points represented one trial with duplicate plating.

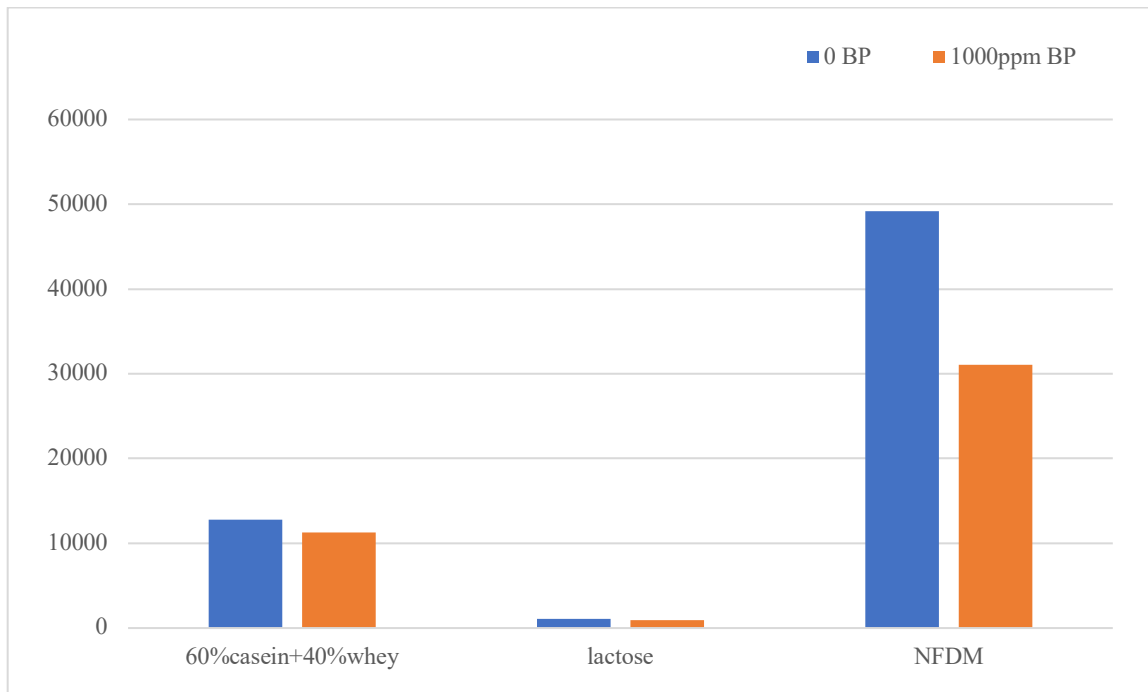
4.2.1.3.3 Fluorescence study of potential binding between butyl paraben and PIF.

Measuring the fluorescence of BPB was an additional tool for determining which component that was an auxiliary test to determine if BPB was binding to a component of NFDM or PIF. When a chemical entity binds to BPB, its fluorescence is diminished, and if not, the fluorescence remains unchanged. NFDM mainly contains approximately 50% of lactose and 50% of protein by weight. Each of the substance was dissolved in water to measure the fluorescence. The concentration of each isolated content in NFDM was calculated based on the full serving size of NFDM, and the fluorescence was measured on each group with and without 1000 ppm of BPB. A mixture of 60% casein and 40% whey protein was used to represent protein profile in PIF and NFDM. The fluorescence results were shown in Fig. 4-12. The results in Figure 4-12 represented one run of experiment with triplicate measurement, which was not enough to perform a complete statistical analysis but was clear enough to show the general response. The large fluorescence difference

observed with NFDM between 0 ppm control and 1000 ppm BPB demonstrated that BPB was effectively binding to some fluorescent substance(s) in NFDM. The mixture of casein and whey proteins showed slight fluorescence, but the quenching by BPB was limited, likely due to the low solubility of these two proteins. On the other hand, lactose did not appear to bind BPB to any degree. As a result, some substances in NFDM, most likely proteins, were effectively binding to BPB, which was likely blocking its synergistic antimicrobial effect with mild heating. This suggests that successful potential food processing applications would likely be foods that do not contain large amounts of proteins.

Overall, the data suggest that the lack of enhanced thermal inactivation by BPB in PIF and NFDM was due to its interaction with the protein components.

Figure 4-11: Fluorescence test results on isolated content of NFDM with and without 1000 ppm BP.



* Results represented one replicate.

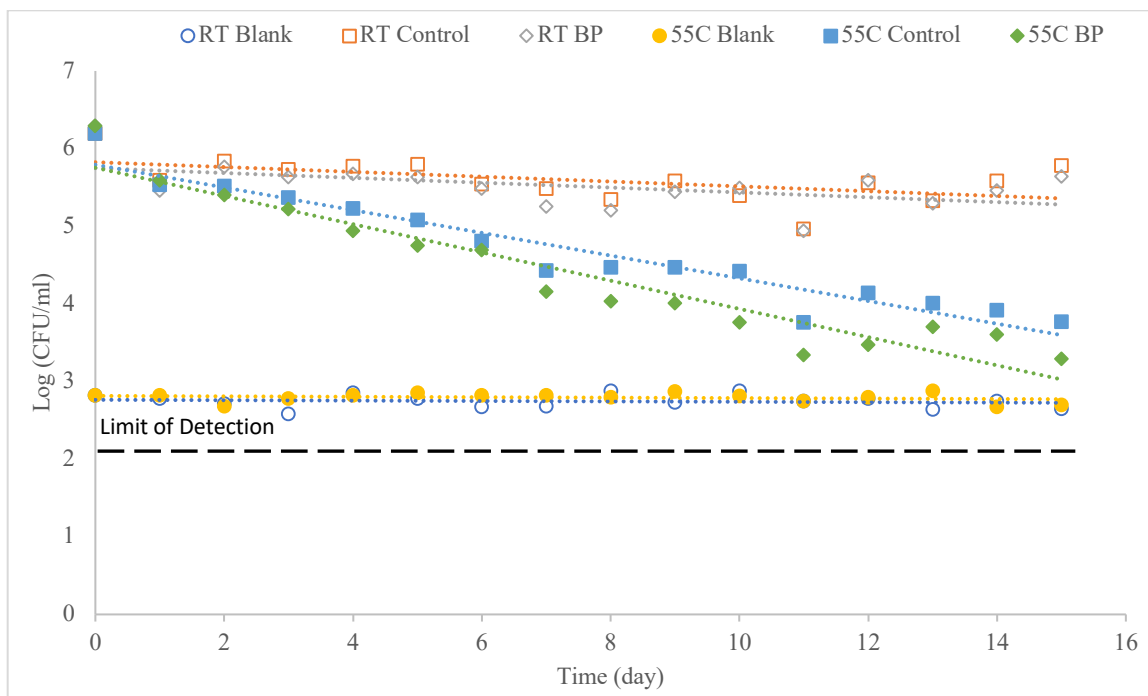
4.2.2 NFDM study

Previous experiments on rehydrated PIF indicated that the proteins in PIF and NFDM quench thermal enhancing activity of BPB when powder dissolved in water. Aqueous phase might help proteins solubilize and interact with BPB. A second NFDM study was undertaken to assess the potential application of BPB in dry powders held at an elevated temperature (55 °C) for 15 days. NFDM was inoculated with *C. sakazakii*, and BPB added to a level of 125 ppm in final powder product. The water activity of (a) NFDM controls without inoculation or BPB inclusion, (b) NFDM inoculated with *C. sakazakii* but not containing BPB, and (c) NFDM inoculated with *C. sakazakii* and BPB all had a_w values in the range of 0.25 to 0.30 without significant differences. The microbial loads generally followed first order log-linear inactivation kinetics (Fig. 4-12). The background microbiota was low and remained unchanged at both room temperature and 55 °C for 15 days. The level of *C. sakazakii* in the inoculated samples that were held at 55 °C declined by less than 3 logs over the 15-day, while the NFDM held at room temperature declined by approximately 0.5-log. There was no difference in the rate or extent of *C. sakazakii* inactivation at room temperature in the presence or absence of 125 ppm BPB. At 55 °C, the rate of inactivation was slightly greater in the NFDM containing BPB than the corresponding NFDM without BPB, however, the difference was minimal. The data indicated that the addition of BPB to dry NFDM did not offer enhanced thermal inactivation.

The results were somewhat confounded by the observation that over the course of the 15-day 55 °C treatment, the a_w dropped from an initial level of approximately 0.25 to 0.045 in all three groups over the 15-day, 55 °C storage. The decrease in a_w would be expected to increase the thermal resistance of *C. sakazakii*. Whether this had any practical impact would require

future trials where samples are thermally treated in sealed containers to prevent the further desiccation of the NFDM.

Figure 4-12: Survivor curves of *C. sakazakii* in NFDM powders with different treatments for 15 days



* All of points represented one replicate.

4.3 Apple juice studies

The prior experiments indicated that BPB was not effective before or after rehydration of dry foods with high protein content, despite the clearly significant thermal inactivation enhancement resulting from the synergistic effects of between low levels of BPB and mild heating in the model system studies. This suggested that this phenomenon may have beneficial food applications in foods that are relatively low in protein. As an example of such a food,

commercial apple juice made from apple juice concentrate was selected to assess the potential bactericidal enhancement resulting from BPB and mild thermal treatments.

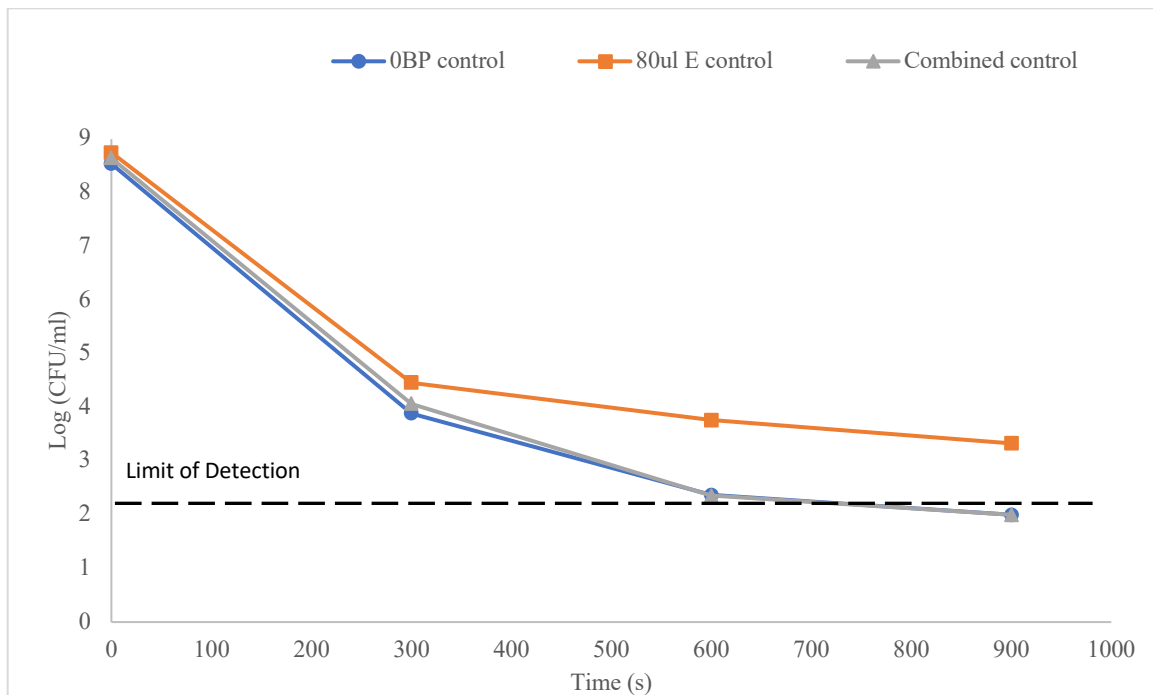
The primary components of clarified commercial apple juice are quite simple, whether freshly pressed or processed from concentrates. The major components of apple juice from concentrates are filtered water, apple juice concentrate, natural flavors, malic acid and ascorbic acid. (This information was obtained from the ingredient label of the brand of commercial apple juice used to conduct this study.) Apple juice does not contain notable amounts of protein or other complex organic molecules, so it was hypothesized that it would not inhibit BPB. One additional important characteristic of apple juice was its acidity (pH range = 3.35 to 4.00), with malic acid as its primary organic acid. The mean pH observed with the commercial apple juice used in these studies was 3.80. This pH/acidity would be expected to enhance microbial inactivation during thermal processing. This enhancement was likely to be due to apple juice's low pH and possibly malic acid or ascorbic acid or the combination of both pH and organic acid anion. As a result, experiments were designed to investigate the enhancement of thermal inactivation by both BPB and malic acid in apple juice and the influence of pH on BPB and malic acid bactericidal effects. In addition, the effect of malic acid at low pH on microbial inactivation and its potential synergism with BPB. All of experiments with apple juice were conducted with *C. sakazakii* at 58 °C.

4.3.1 Room temperature and 58 °C controls

The pH value of apple juice used in the study was approximately ranged from 3.65 to 3.85, and all juice used for these studies was from the same lot. For purposes of the following experiments, the original pH of original apple juice was assumed to be 3.80. It was

assumed that the pH/acidity of apple juice would have some degree of bactericidal effect without inclusion of BPB. As before, the BPB was dissolved in 80 µl of 70% ethanol, so ethanol only controls of 80 µl of 70% ethanol were included. There was no significant differences between 0 ppm BPB controls and ethanol only control groups. For subsequent trials, the average values of BPB control group and ethanol control group were used as the general control group of *Cronobacter sakazakii* 607 in original pH apple juice at 58 °C throughout the experiments (the “combined control” in Figure 4-13).

Figure 4-13: Survivor curves of controls of *Cronobacter sakazakii* 607 in original pH apple juice at 58 °C.

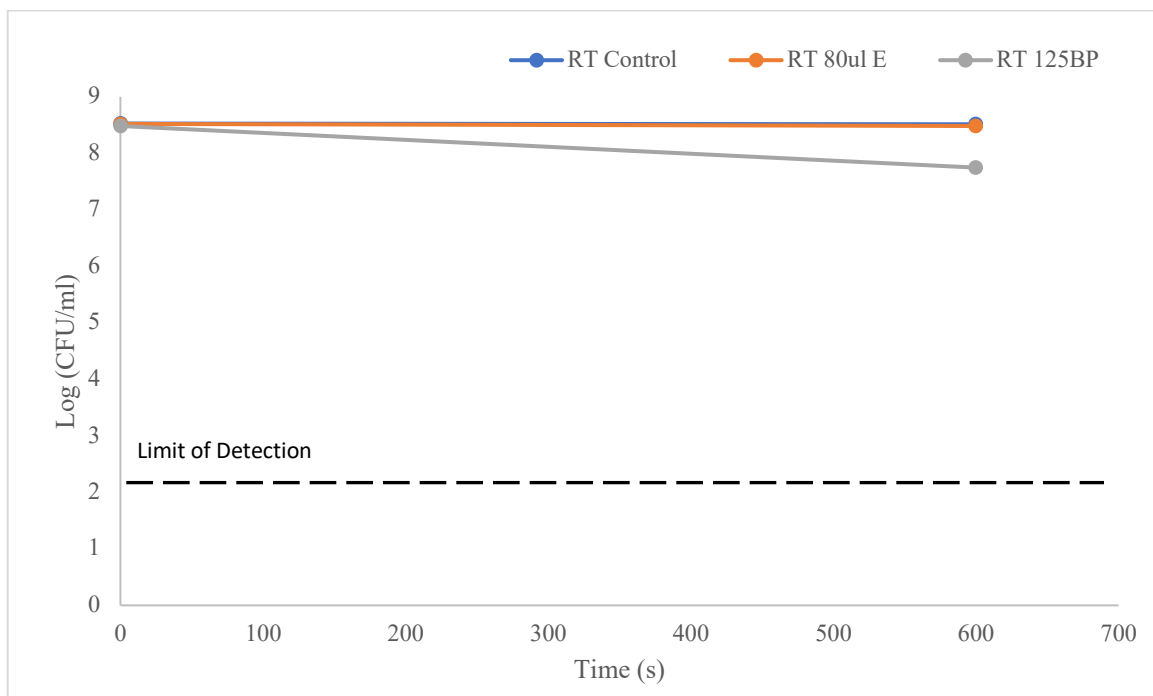


* All of points represented three replicates.

The apple juice BPB experiments started with room temperature controls trials (Fig. 4-14). During the 10 min treatment, *C. sakazakii* levels remained unchanged in both apple juice

only and apple juice with 80 µl of 70% ethanol. The 80 µl of 70% ethanol used to solubilize BPB did not change the survival pattern of *C. sakazakii* in apple juice at room temperature. Less than a 1-log reduction in *C. sakazakii* was observed at room temperature in apple juice with 125 ppm BPB. Thus, 125 ppm BPB was not an effective antimicrobial at room temperature after a 10 min exposure, despite the low pH of the apple juice.

Figure 4-14: Survivor curves of *C. sakazakii* with BPB or ethanol at room temperature.

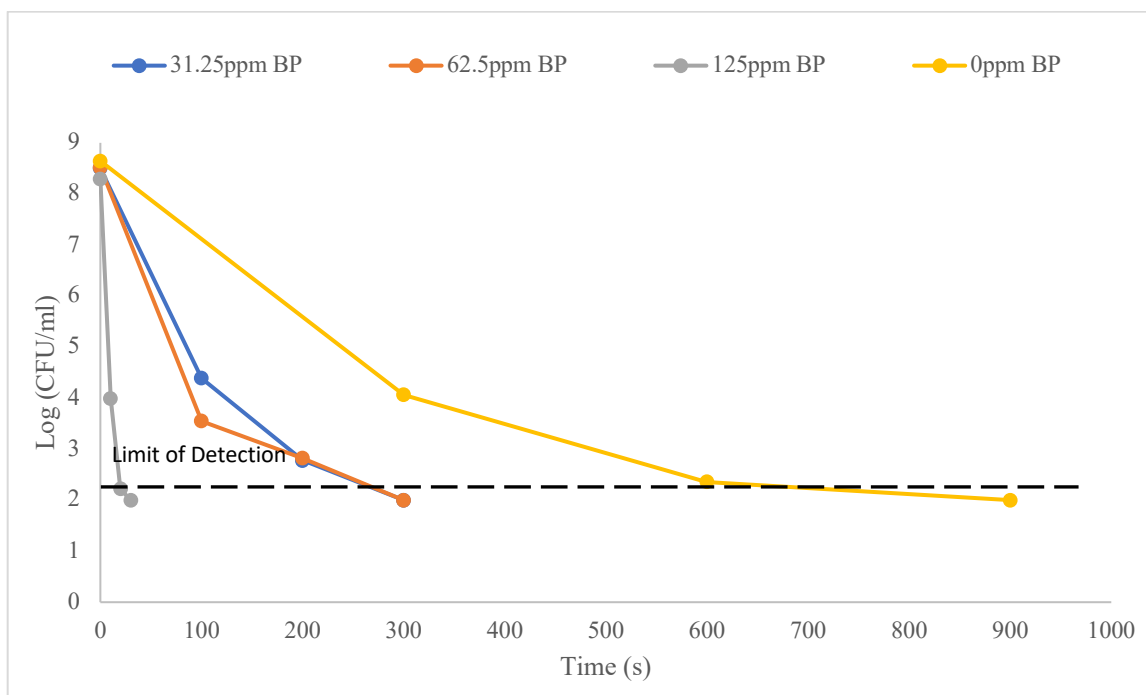


* All of points represented three replicates.

4.3.2 Evaluation of the effect of BPB on the thermal inactivation of *C. sakazakii* in apple juice at pH 3.8

Apple juice was inoculated with a 24-h *C. sakazakii* culture and designated levels of BPB was added just prior to the start of a thermal trial, using the submerged coil apparatus at 58 °C. The initial trials evaluated the effect BPB effect at the original apple juice pH (3.80) (Fig. 4-15). The BPB enhanced the effect mild heating in a concentration dependent manner (Fig. 4-15). The 0 ppm BPB controls heated at 58 °C achieved more than 6-log reduction in 600 seconds whereas addition of 125 ppm BPB reached 6-log reduction in less than 30 seconds. Because the microbial populations decreased so rapidly, calculation of D-value could only be considered estimates. Both the 31.25 ppm and 62.5 ppm groups showed similar patterns, reaching a 6-log reduction within 300 seconds. Lack of sampling point between 200 seconds and 300 seconds created difficulty to determine the exact time to finish 6-log reduction, but they were significantly different from 0 ppm group ($P < 0.05$). It is clear that low levels of BPB were highly effective at enhancing inactivation of *C. sakazakii* in apple juice at 58 °C.

Figure 4-15: Survivor curves of *C. sakazakii* in pure apple juice at 58 °C with different levels of BP.



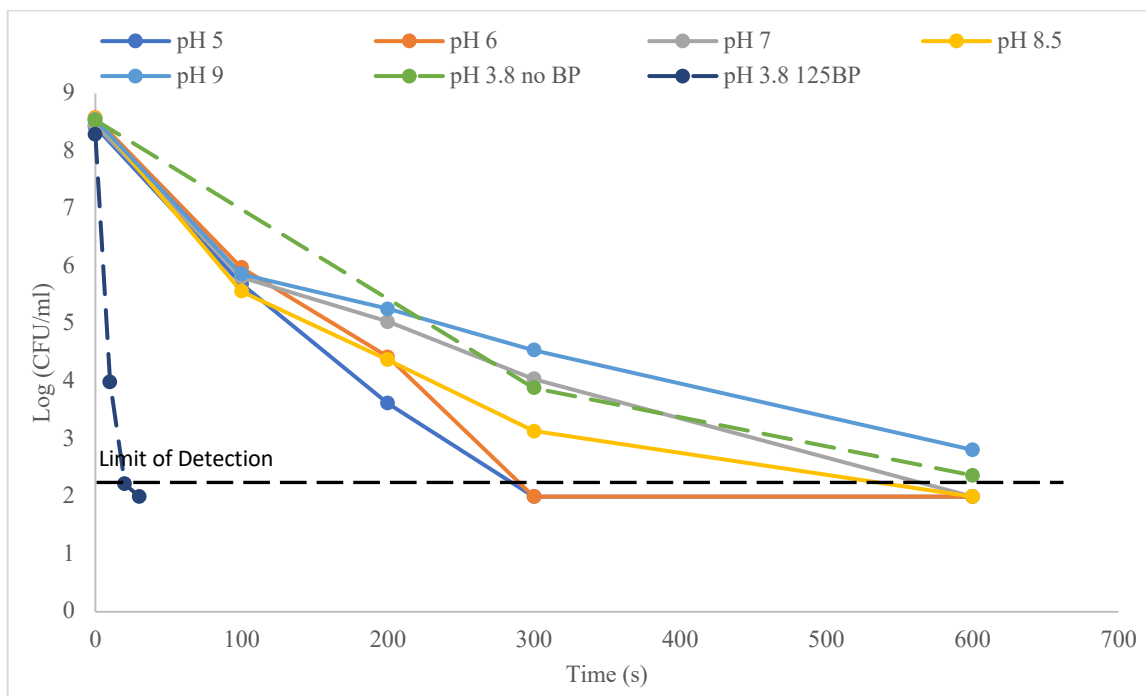
* All of points represented three replicates.

4.3.3 Investigation of effect of pH on the synergistic effect of BPB and heating at 58 °C on the inactivation of *C. sakazakii* in apple juice

Apple juice adjusted to pH 5.0, 6.0, 7.0, 8.5, and 9.0 was used as the experimental matrix to determine the effect of pH on the enhancement of thermal inactivation at 58 °C by 125 ppm BPB (Fig. 4-16). The pH 8.5 value was specifically chosen because the pK_a of BPB is 8.48. As the pH values increased, the synergism of BPB generally decreased. Compared to pH 3.8/125 ppm BPB conditions, survivor curves at higher pH values displayed greater heat resistance, i.e., significantly higher D-values. In general, biphasic survivor curves were observed, with rapid bacterial inactivation during the first 100 sec in the pH 5 to 9 trials. After 100 sec, the slopes of the survivor curves decreased in a largely pH dependent manner. Particularly noteworthy is a comparison of the pH 3.8/0 ppm BPB survivor curve and that of the pH 9.0/125 ppm BPB survivor curve which were roughly equivalent. This suggests that at pH 9 the BPB has lost all of

its ability to enhance thermal inactivation of *C. sakazakii*. At pH 9, BPB should largely be in dissociated form and therefore be no longer able to penetrate the cell membrane.

Figure 4-16: Survivor curves of *C. sakazakii* in pure apple juice with adjusted pH at 5, 6, 7, 8.5, and 9 with inclusion of 125 ppm BPB at 58 °C

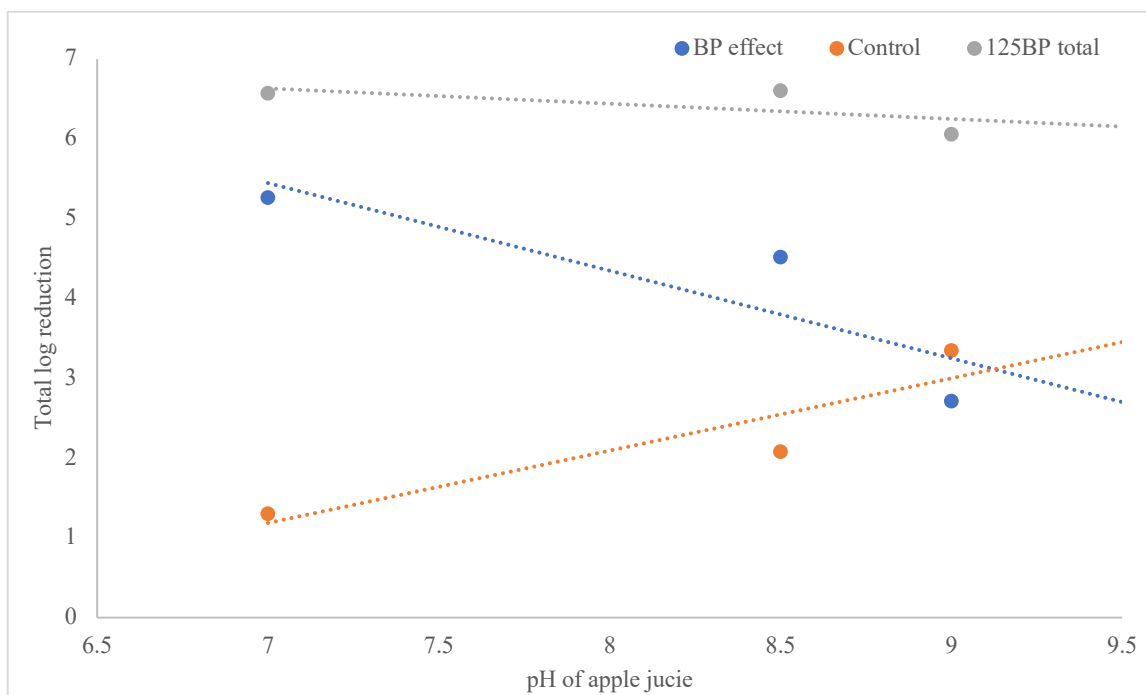


* All of points represented three replicates.

The Henderson-Hasselbalch Equation ($\text{pH} = \text{pK}_a + \log\left[\frac{A^-}{HA}\right]$) was used to calculate the percentages of BPB that would be in the undissociated form. At pH 5.0, 6.0, 7.0, 8.5 and 9.0 the percent undissociated BPB would be 99.97%, 99.67%, 96.73%, 48.28%, and 22.79%, respectively. Survivor curves of pH 5 and 6 shared highly similar inactivation pattern and this similarity generally agreed with the ratios of undissociated form of BPB. Based on the combined results of apple juice survivor curves at pH 5 to 9 with 125 ppm BP, we studied the survival kinetics at pH 7.0, 8.5 and 9.0 in more detail. This pH range brackets the pK_a transition of BPB.

We then tested survivor kinetics at pH 7, 8.5 and 9 with and without 125 ppm BPB. (Survivor curves not shown). Using the regression analysis to calculate the slopes and derive D-values for comparison did not provide useful insights. Alternatively, a comparison on the total log reduction achieved in 600 seconds for each treatment combination provide useful. The total reduction for the 0 and 125 ppm BPB trials were plotted at the three pH values (Fig. 4-17). To assess the “BPB effect” the degree of microbial reduction (log CFU/ml) from the apple juice without BPB was subtracted from the microbial reduction in the apple juice containing 125 ppm BPB (Fig. 4-17). The total reduction of control groups in 600 seconds increased as the pH increased whereas the 125 ppm BPB groups have highly consistent total log reductions at all three pH values. These results suggested that at pH values ≥ 7.0 the lack of BPB enhancement is offset by decreased heat resistance of *C. sakazakii* as the pH becomes more alkaline. This suggested that future research should consider expanding these data to even more basic pH levels.

Figure 4-17: Total log reduction of *C. sakazakii* in apple juice at pH 7-9 with and without 125 ppm BP at 58 °C in 600 seconds

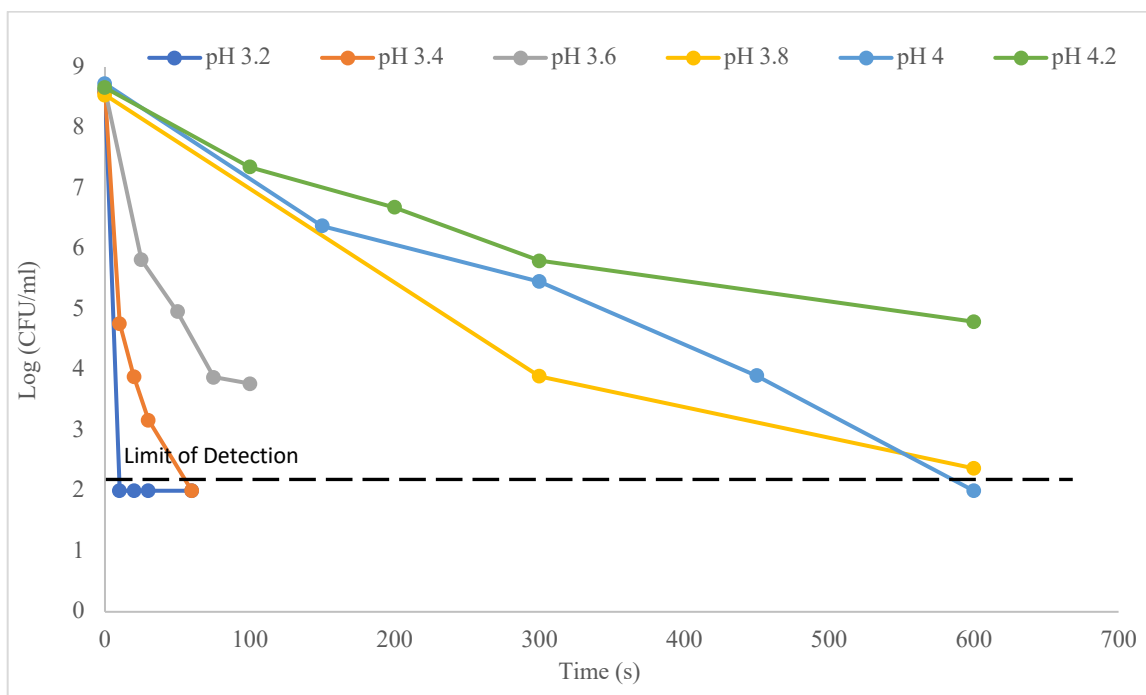


* All of points represented three replicates.

4.4.4 Investigation of synergism in apple juice at pH below 5.0 with additional MA

After the investigation of apple juice at pH higher than 5, we also conducted trials at a pH range from 3.2 to 4.2 (Figure 4-18). At pH 3.2, the strong acidity eliminated the microbial population from more than 8.5 logs to below lower detection limit in 10 seconds. And this level of inactivation for pH 3.4 group required 60 seconds. Because we stopped sampling pH 3.6 group at 100 seconds, we could only conclude that 5-log reduction required at pH 3.6 was around 100 seconds. The inactivation rates of pH 3.8 and pH 4 groups were similar, and pH 4.2 group only achieved a 4-log reduction in 600 seconds. In general, the survivor curves of pH 3.2 to 4.2 showed clear trend that as the pH declined, the thermal inactivation significantly enhanced.

Figure 4-18: Survivor curves of *C. sakazakii* in apple juice with 0 ppm BPB at 58 °C adjusted pH range from 3.2 to 4.2.

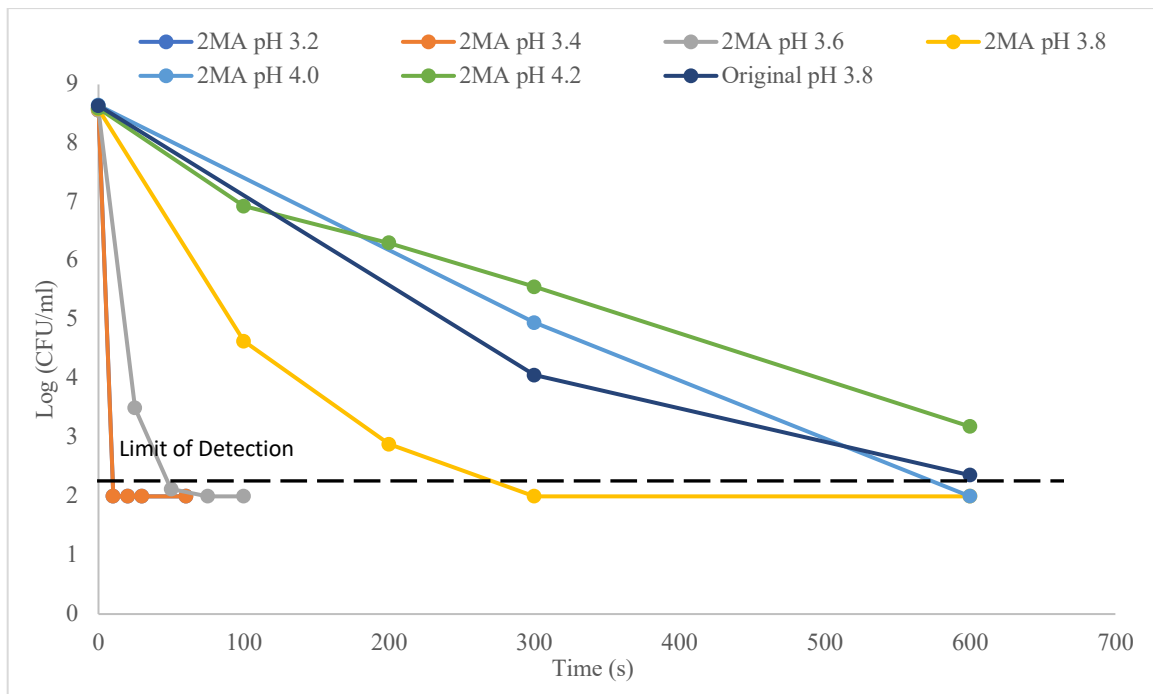


* All of points represented three replicates.

Another organic compound that might be influencing microbial inactivation was malic acid (MA). According to Krueger Food Laboratories (Krueger Food Laboratories, 2019), the median concentration of MA in commercial apple juice is 0.45 g MA/100 g apple juice, with most samples falling within the range of 0.3 and 0.6 g/100 g. To investigate the potential synergism among BPB, MA, and mild heating processing, apple juice with additional 0.5 g MA/100 g was used as liquid matrix to test the survivor kinetics of *C. sakazakii* at 58 °C. (Figure 4-19). This liquid matrix was considered as 200 % (2X) MA apple juice. The additional 0.5g MA/100 g dropped the pH of apple juice from 3.8 to 3.2, and the pH had to be subsequently adjusted with 1N NaOH solution to 3.2 to 4.2 to invest the MA effect on microbial inactivation kinetics. The 2X-MA apple juice at pH 3.2 and 3.4 both reached more than 6-log reduction within 10 seconds at 58 °C, and 2X-MA apple juice at pH 3.6 achieved more than 6-log reduction within 50 seconds at 58 °C, which was faster than the 1X-MA control group at pH 3.6.

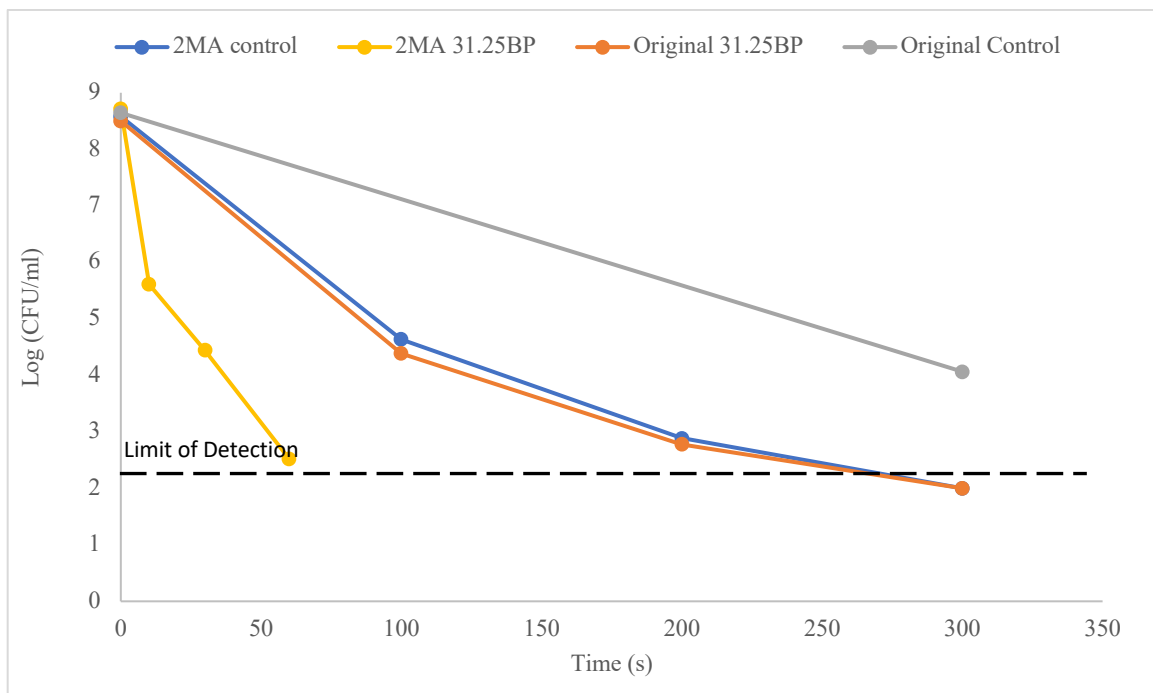
The time required to achieve a 6-log reduction in original apple juice at pH 3.8 with inclusion of 125 ppm BPB was within 30 seconds. The inactivation rates of all these four groups were considerably fast, and 125 ppm BPB showed similar extent of antimicrobial effect compared to 2X-MA groups at pH values below 3.6 without BPB. However, BPB was used at an extremely lower concentration level and it would not change the pH of matrix. On the other hand, if we compare 2X-MA group at pH 3.8 and original apple juice group at pH 3.8, we could clearly conclude that MA significantly increased microbial inactivation rate. The additional 0.5g/100g of MA reduced the time required for 6-log reduction from 600 seconds to less than 300 seconds. When the pH of apple juice raised to 4.0 and 4.2, the addition of MA did not induce synergism on microbial inactivation compared to original apple juice at pH 3.8. Based on their survivor curves, all three conditions required around 600 seconds to achieve a 6-log reduction.

Figure 4-19: Survivor curves of *C. sakazakii* in apple juice with additional 0.5g/100g MA at adjusted pH range from 3.2 to 4.2 at 58 °C



To further compare the synergism induced by MA and BP, low level of BPB was added to 2X-MA apple juice that the pH was already adjusted back to 3.8. And the survivor curves prior to 300 seconds were plotted in Figure 4-20 for the comparison between original apple juice and 2X-MA apple juice. With additional 0.5g MA/100g apple juice, the inactivation rate increased to the same level of original apple juice with 31.25 ppm BPB. These two survivor curves almost perfectly overlapped, which means the inactivation rates were same. Thus, in the apple juice added with 0.5g MA/100g, adjusted the pH back to 3.8 and supplemented with 31.25 ppm BP, there was a clear synergistic interaction between MA, BPB, and mild heating, i.e., the additional portion of MA helped decrease the time required to achieve 6-log reduction from 300 seconds to 60 seconds.

Figure 4-20: Survivor curves of *C. sakazakii* in apple juice with additional 0.5g/100g MA and inclusion of 31.25 ppm BP at pH 3.8 at 58 °C



Chapter 5: Discussion, Conclusions, Recommendations for Future Research and Potential Impact of Study

5.1 Discussion of study

Approved preservative, BPB, was investigated for their bactericidal and fungicidal properties, including the synergistic effects of BPB with mild heating in different models and food matrices. In general, the addition of low levels of BPB significantly enhanced the thermal inactivation at mild heating temperatures.

5.1.1 Brain and heart infusion broth study

This study examined the potential synergistic effect on inactivation of four foodborne pathogenic bacteria, *Cronobacter sakazakii* 607, *Salmonella enterica* serotype Typhimurium, attenuated *Escherichia coli* O157:H7 and *Listeria monocytogenes*, during mild heating by inclusion of BPB in BHI model matrix. The results indicated that low levels of BPB produced a significant synergistic enhancement of the thermal inactivation at mild heating temperatures. Minimal use of ethanol as a solubilizing agent generally did not affect the microbial inactivation. Microbial levels in room temperature controls with and without addition of BPB generally remained unchanged during treatment, demonstrating that BPB and mild heating were acting synergistically. Addition of paraben can significantly enhance the rate of thermal inactivation of foodborne pathogens, which may enable mild heating temperatures or shorter processing times. This study provides a proof of concept that identification of synergistic processing agents could be a means of enhancing the lower temperature thermal processing.

The only Gram-positive microorganisms tested in this study was *L. monocytogenes*, and it showed a different inactivation pattern than the other three gram-negative bacteria. The inactivation of *L. monocytogenes* was a two-phase process including a relatively rapid death at

the beginning of the heat treatment, followed by a slow decrease in the second phase. The transition point was approximately at 300 seconds. This type of two-phase inactivation modes was observed both in control group (the treatment without any chemicals) and BPB treatment groups. These survivor kinetics curves suggested that there was a subpopulation that was quickly injured and inactivated. But the underlying reason that this phenomenon only occurred in *L. monocytogenes* remained undiscovered. We did not test other Gram-positive strains in this study, so we cannot conclude this two-phase kinetics was only associated with *L. monocytogenes* or it was the characteristic of Gram-positive strains. *Listeria monocytogenes* also showed high resistance to the combined of BPB and mild heating treatment. The highest concentration at 125 ppm butyl parabens combined with 54 °C heating only produced approximately 3.5-log reduction to *Listeria monocytogenes* in 15 minutes. One of the possible explanations is that gram-positive organisms have a different cell structure than gram-negative organisms. In gram-positive microorganisms, peptidoglycan (PG) accounts about 40% or more of the mass of cell wall, and it contains one or more (depending on species) non-PG (or accessory) wall polymers, such as teichoic acids (TAs), teichuronic acids (TUAs), and/or neutral or other acidic poly saccharides (Shockman and Barrett, 1983). The thick PG layer and teichoic acids might have blocking effect that prevent BPB getting through the cell wall and cell membrane. We estimated that the structural difference was most likely the reason that *L. monocytogenes* showed the most resistance to the treatment. However, our results did not agree with Eklund's study (1980) and Freese's study (1973): they stated that gram-negative bacteria were, most likely, resistant to the parabens owing to a screening effect by the cell wall lipopolysaccharide layer. Additional future studies will be necessary to determine the underlying principle of this phenomenon.

5.1.2 PIF and NFDM study

With the knowledge of the synergism between mild heating and BPB on the inactivation of these four pathogens, the study extended the application of this treatment on *C. sakazakii* to dry powder matrix powdered infant formula (PIF) and non-fat dry milk (NFDM). The overall experimental results indicated that BPB was not effective on enhancing *C. sakazakii* thermal enhancement in PIF or NFDM in both rehydrated form and dry powder form. PIF contains large portion of lipid contents and BPB is usually easily to solubilize in lipid portion rather than aqueous portion. In rehydrated PIF study, the effectiveness might be limited because BPB simply precipitated from liquid mixture or partition into lipid portion in PIF. In order to reduce the thermal resistance of bacteria cells, BPB must have direct contact with the cells, and either of the cases allowed this type of contact. Heated water with minimal amount of DMSO was used to rehydrate PIF to help the water solubility issue. (Data not shown). The results showed no noticeable difference from regular water group at the same condition. However, there was no efficient test to examine whether the addition of DMSO will completely solve the problem of BPB precipitation in PIF rehydrating water. On the other hand, NFDM was selected to substitute PIF to avoid the partitioning issue associated with high fat content in PIF. According to Figure 4-9, rehydrated full serving size of NFDM completely inhibited BPB's effect on bacteria inactivation at 58 °C: it achieved approximately 2-log reduction in 600 seconds, and it was consistent with the control group in BHI study at 58 °C. The fluorescence test results revealed the interaction between BPB and NFDM, and the interaction was most likely only occurred with proteins in NFDM but not the lactose. Survivor curves of *C. sakazakii* in BHI with 125 ppm BPB and addition of different percentages of NFDM or lactose at 58 °C also confirmed the results

from fluorescence test. In conclusion, the application of BPB in food processing are not likely to be effective in foods that contain high fat and/or protein contents.

To minimize the partition of BPB into fat content and the contact between BPB and proteins in rehydrated PIF, low level of BPB was added in dry NFDM powder in which the protein would not flow around. Although the results indicated BPB was not effective in NFDM powder at 55 °C, we could not simply conclude the underlying mechanism underlying this effect. It might also cause by the interaction between proteins and BPB, or the lack of contact between BPB contact with the bacteria cells, so it could not disrupt the cell membrane permeability. Sampling method on detecting the microbial loads in NFDM involved a rehydration step at room temperature. Considering the insolubility of BPB and the low water temperature, the contact between BP and bacteria cells in sampling step was likely negligible.

Another parameter associated with PIF and NFDM study was water activity. Microorganisms in low-moisture environments are in general more tolerant to heat (Syamaladevi et al., 2016). The current experiment did not control water activity over the complete time course of 15 days: the water activity of samples stored at 55 °C dropped from approximately 0.240 to 0.045 in 15 days, while the room temperature groups remained unchanged. The samples were stored in petri dishes without completely sealed but this condition was close to the real-life cases. This setting introduced a parameter that was changing during the experiment. So, we could not conclude the 3-log enhanced reduction observed in elevated temperature groups was only resulted from the temperature difference.

5.1.3 Apple juice study

The only liquid food system tested in this study was apple juice. Low pH, low levels of MA and BPB all could significantly enhance the *C. sakazakii* inactivation at 58 °C. The results also proved that parabens had a wider effective pH range compared to benzoic acid (Aalto et al., 1953). In the comprehensive pH study on the effectiveness of BPB, 125 ppm BPB at pH above 5 showed significant difference compared to 125 ppm BPB at pH 3.8. Although pKa of BPB was 8.48, results showed that 125 ppm BPB could significantly strengthen the inactivation even at pH 9 condition, achieving 6-log reduction in 600 seconds. Compared to the control groups at pH 7-9, 125 ppm BP were still effective in a limited manner on enhancing *C. sakazakii* inactivation at 58 °C in all three pH values. But the enhancement induced only by BPB weakened as the pH increased. According to the Henderson-Hasselbalch equation, the undissociated form of BP amount substantially decreased as the pH increased to the pH around pKa. And the results generally agreed with this hypothesis.

There was only one set of experiment examined the relationship between malic acid and BPB in thermal inactivation (Figure 4-20), and we concluded with an additive relationship. However, more data is needed define if the enhancement on thermal inactivation resulted from two chemicals was definitively additive rather than synergistic. This experiment was conducted only at pH 3.8, so it also excluded the potential effect of pH on the relationship between two chemicals.

5.2 Conclusion

In conclusion, BPB was generally efficient on assisting the thermal inactivation at mild heating temperatures in food does not contain extensive amount of protein and lipid contents. In

BHI study, *C. sakazakii* 607, attenuated *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* were all observed significant enhancement on thermal inactivation with low levels of BPB (<125 ppm) at temperatures no more than 58 °C. And the enhancement was completely induced by BPB. PIF and NFDM studies indicated BPB was efficiently interacting with some component of PIF and NFDM, most likely proteins. And this interaction completely inhibited the effect of BPB. In apple juice, the acidity also facilitated the synergism between BPB and mild heating, and the synergism reduced as the pH increased from 3.8 to 9. At the same time, malic acid in apple juice also drastically assisted thermal inactivation and the synergism between BPB and mild heating inactivation, which indicated apple juice would be a suitable food example for BPB application in thermal processing.

5.3 Recommendations for future research

In BHI study, the results indicated that *L. monocytogenes* was the most resistant strain against BPB at mild heating. It might be resulted from the gram-positive cell structure properties or it was the special characteristics of *L. monocytogenes*. Using other gram-positive microorganisms and other *Listeria* strains such as *L. innocua*, *L. ivanovii*, and *L. seeligeri* to perform the same treatment would be a proper choice to confirm the hypothesis. And it can be proved by comparing the antimicrobial activities under the same treatment condition and observing the membrane transport under electron microscopy combined with fluorescent staining, which would be a proper future experiment. Another possible future study is to use the cocktail strains of each of four bacteria and perform the same set of treatments combined with PCR and DNA polymorphism analyses. The strains tested in this study are the typical representative strains or the most heat-resistant strains, but the resistance may vary among

different strains within the species. Therefore, an alternative future study will be using the cocktail of various clinical isolates to test the resistance to BPB. If the difference occurs, we can also use molecular techniques to investigate the gene expression differences and potentially identify the genetic characterization of the resistance to this combined treatment.

Based on the chemical structure of BPB, it was easily dissolved in lipid but nearly insoluble in water at room temperature. We used NFDM to eliminate the partitioning problem associated with fat content. However, it will be more definitive if there were studies on estimating the BPB level in lipid portion. There will be difficulties on isolating the lipid content from food matrix and maintain the substances in lipid. But wisely designing an experiment to test the BP concentration in lipid will be a proper future research direction.

Water activity was not strictly controlled in NFDM study, and it restricted us from making definitive conclusion that the enhanced thermal inactivation was completely from elevated temperature. To solve this problem, it will be beneficial to conduct a similar experiment that monitor the microbial population in sealed package with controlled water activity. With consistent water activity, we will be able to isolate and examine the potential synergism of BPB at elevated temperatures in dry powder foods.

The apple juice study revealed the potential pH effect on BPB synergism. Therefore, conducting the experiment at higher pH values (above 9) will be supportive to assess the quantitative relationship between the synergism and undissociated form of BP. Malic acid in apple juice also enhanced the microbial inactivation. And it needs more experiments with different levels of MA and BPB in wider pH range to determine if there is additive or synergistic antimicrobial effect between MA and BPB in thermal inactivation. At the same time, apple juice contains low level of ascorbic acid that might affect the results. Therefore, analyzing the

concentration of ascorbic acid and their potential effect on thermal inactivation will also be a recommended future study.

5.4 Potential Impact of Study

BHI study could serve as a model system to study the effect of BPB combined with mild heating against these four microorganisms. The data collected from this model system would provide some fundamental understanding of their resistance to butyl paraben during thermal processing. And those fundamental studies will help develop the potential preservative formula in food and innovative processing approaches.

A section of this study was to apply the treatment onto foods and collect some primary experimental data on how feasible the treatment is during food processing. And the data would provide some understanding on both dry food and liquid food. The tests on the pH effect of this treatment also offered an overview of how the food pH will change the efficiency of this treatment.

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